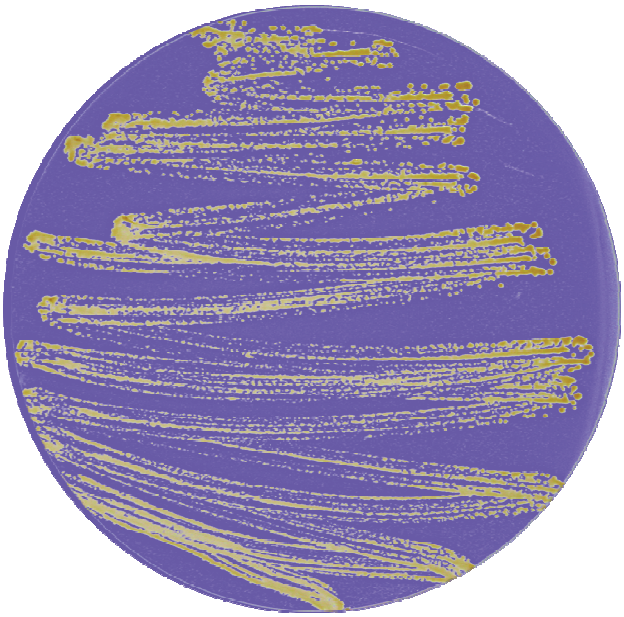
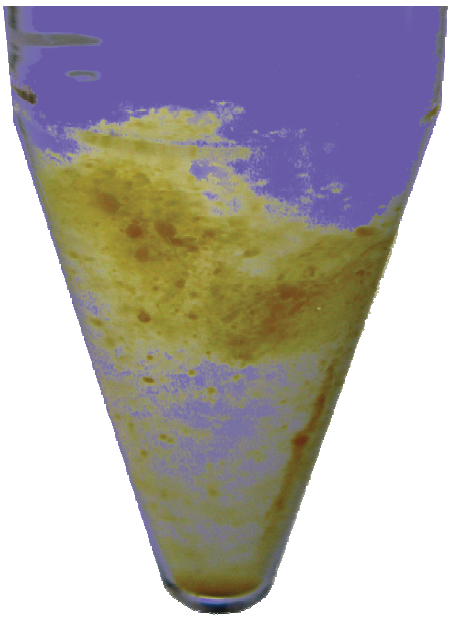
Cultivation of *Flavobacteria* and other *in situ* abundant bacteria from the North Sea





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Cultivation of *Flavobacteria* and other *in situ* abundant bacteria from the North Sea

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For my Family

"But if in your thought you must measure time into seasons, let each season encircle all the other seasons, And let the to-day embrace the past with remembrance and the future with longing."
Khalil Gibran, 1926 In: *The Prophet*

Summary

The **isolation and cultivation of heterotrophic marine bacteria** opens possibilities to study their physiology and genomes with respect to their function in the marine environment. In the pelagic marine realm bacteria remineralize more than half of the photosynthetically produced biomass, and thus play an important role in the biogeochemical cycling of elements.

Flavobacteria are abundant of up to 30% in the North Sea. In previous studies marine *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* were predominantly cultivated, but cultures of *Flavobacteria* were infrequently obtained. This thesis addresses the isolation of **phylogenetically diverse marine *Flavobacteria* using three new approaches**. First, samples were retrieved from various pelagic and benthic habitats of the North Sea. Second, a **new marine artificial seawater HaHa medium** was developed to facilitate the growth of *Flavobacteria*. This medium was supplemented with saccharides and proteins as carbon sources at a concentration of 2 g/L. Third, a specific **16S rRNA gene PCR assay was applied to identify *Flavobacteria-Cytophagia*** among the colonies. The molecular screen was preferred over the identification by cell and colony morphology, since the latter has predominantly resulted in the isolation of strains of the genera *Arenibacter*, *Cellulophaga* and *Maribacter*. The **375 *Flavobacteriaceae* strains** isolated on agar plates comprised (i) seven presumably **novel genera**, (ii) 42 presumably **novel species** in 22 validly described *Flavobacteriaceae* genera and (iii) many isolates that were so far not distinguishable from **37 type strains** in 16 genera. Thus, in contrast to previous studies, we could show that phylogenetically diverse *Flavobacteria* from the North Sea can be cultivated on solid medium.

The **isolation of representative strains of the genera *Formosa*, *Polaribacter*, and *Reinekea* from the North Sea** was attempted. In previous studies these bacterial populations were proposed to be of importance during coastal diatom-dominated phytoplankton blooms, based on their high abundance of 15% to 25% of the bacterioplankton and their potential capability to decompose algae derived polysaccharides. A new medium was devised which had the same composition as the marine HaHa medium, but with **environmental-like** micromolar carbon, nitrogen, and phosphate **concentrations**. **Aerobic dilution cultivation** in the HaHa medium led to a high **culturability of 35% of the bacterioplankton in spring 2010** and 27% of the bacterioplankton in summer 2010. **23 strains of *Flavobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Actinobacteria*** were obtained directly by dilution cultivation of single cell inocula. One strain that belonged to the genus *Reinekea* was isolated by generating co-cultivatures of **randomly mixed bacterial populations** which potentially had a positive effect on the growth of *Reinekea*. Strains that affiliated with *Polaribacter*, *Formosa*, *Gillisia* (*Flavobacteria*), the *Roseobacter* clade associated (RCA) lineage (*Alphaproteobacteria*), *Reinekea*, and the OM182 clade (*Gammaproteobacteria*) had **16S rRNA gene sequence identities of >99.9%** with 16S rRNA clones of the bacterioplankton from the North Sea in spring 2009. In addition, draft genomes of *Formosa*, *Polaribacter*, and *Reinekea* strains were used to recruit reads of metagenomes of the bacterioplankton in spring 2009. Thereby, reads of >95% nucleotide identity **covered the draft genomes** of the *Formosa* clade B strain to **94%**, of *Reinekea* sp. to **90%** and of *Polaribacter* sp. to 50%. Based on these results we argue that the novel species of *Formosa*, *Polaribacter*, and *Reinekea* are **representatives of ecologically relevant clades** catalyzing the remineralization of coastal diatom-dominated phytoplankton biomass.

The **physiological characteristics** of the strains were investigated focusing on the growth on different mono- and polysaccharides, to provide further evidence that *Formosa*, *Polaribacter* and *Reinekea* species could prevail in different ecological niches during algae decay. Interestingly, *Polaribacter* strains grew heterotrophically on all tested sulfated (e.g. agar, carrageenan) and non-sulfated polysaccharides (e.g. cellulose, laminarin), whereas *Formosa* strains grew only on non-sulfated polysaccharides. In contrast, *Reinekea* sp. did not grow on polysaccharides but on all tested mono-, di-, and trisaccharides including N-acetylneuraminic acid. Finally, I proposed for these **novel species** the **names** '*Formosa flavarachnoidea*', '*Formosa forsetii*', '*Polaribacter forsetii*', '*Polaribacter frigidimaris*', '*Polaribacter adhaesivus*', and '*Reinekea forsetii*'.

"Doch wenn ihr die Zeit in eurem Denken nach Jahreszeiten messen müsst, dann lasst eine jede alle anderen erfassen. Und lasst das Heute die Vergangenheit mit der Erinnerung umfangen und die Zukunft mit der Sehnsucht."
Khalil Gibran, 2002 In: *Der Prophet*

Zusammenfassung

Die **Isolation und Kultivierung von neuartigen marinen Bakterien** ermöglicht es deren Physiologie und Genome im Detail zu studieren, mit dem Ziel ihre Funktion in ihrer natürlichen Umgebung zu verstehen. Im pelagischen Bereich des Meeres verstoffwechseln Bakterien mehr als die Hälfte der durch Photosynthese gewonnenen Biomasse.

Bakterien der Klasse *Flavobacteria* können einen Anteil von bis zu 30% am Pikoplankton in der Nordsee ausmachen. In der Vergangenheit wurden vorrangig marine Bakterien der Klassen *Alphaproteobacteria*, *Gammaproteobacteria* und *Actinobacteria* isoliert, wobei Vertreter der *Flavobacteria* selten in Kultur gebracht werden konnten. **Drei konzeptionell neue Ansätze wurden verfolgt, um phylogenetisch unterschiedliche Vertreter mariner *Flavobacteria* zu isolieren.** Zunächst wurden Proben aus verschiedensten pelagischen und bentischen Lebensräumen entnommen. Weiterhin wurde das **neuartige marine HaHa Medium** entwickelt, bei dem Saccharide und Proteine mit einer Gesamtkonzentration von 2 g/L als Kohlenstoffquellen dienten und welches das Wachstum von verschiedenen *Flavobacteria* begünstigte. Zusätzlich wurde ein ***Flavobacteria-Cytophagia* spezifischer 16S rRNA PCR basierter Test** angewandt, um diese unter den gewachsenen Kolonien zu identifizieren und phylogenetisch zuzuordnen. Dieser Test ersetzte die Pigmentierung und Zellform als Identifizierungskriterien, die anfäglich dazu geführt haben, dass hauptsächlich Vertreter der Gattungen *Arenibacter*, *Cellulophaga* und *Maribacter* isoliert wurden. Die **Sammlung von 375 *Flavobacteriaceae* Isolaten** beinhaltete (i) sieben möglicherweise **neue Gattungen**, (ii) 42 möglicherweise **neue Arten** aus 22 Gattungen und (iii) weiteren Isolate die von **37 Typstämmen** aus 16 Gattungen nicht zu unterscheiden waren. Anhand dieser Resultate konnte gezeigt werden, dass es möglich

ist, eine Vielzahl phylogenetisch diverser *Flavobacteria* aus der Nordsee auf Agarplatten zu isolieren.

Ein weiteres Ziel dieser Arbeit war es, **repräsentative Stämme der Gattungen *Formosa*, *Polaribacter* und *Reinekea* aus der Nordsee** zu isolieren. Aufgrund der bemerkenswerten Zellzahlen von 15% to 25% des Bakterioplanktons und der potenziellen Fähigkeit von Algen stammende Polysaccharide in der Nordsee abzubauen, wurden in einer früheren Studie Vertretern dieser Gattungen eine wichtige ökologische Bedeutung zugeschrieben. Das in diesen Untersuchungen verwendete Medium wies die gleiche Zusammensetzung auf wie das neu entwickelte HaHa Medium, jedoch mit Kohlenstoff-, Stickstoff- und Phosphatkonzentrationen, die **mit den micromolaren Konzentrationen des beprobten Meereswassers vergleichbar** waren. Durch aerobe Verdünnungskultivierung (engl. ***dilution cultivation***) der Wasserprobe in dem **nährstoffarmen HaHa medium** konnte eine **Kultivierbarkeit von 35% des Bakterioplanktons im Frühling und 27% im Sommer 2010** erreicht werden. Weiterhin konnten durch die Verdünnungskultivierung von einem Nanoliter Meereswasser **23 Stämme** kultiviert werden, die den *Flavobacteria*, *Alphaproteobacteria*, *Gammaproteobacteria* und *Actinobacteria* zugeordnet werden konnten. Bei der Isolierung einer neuen Art aus der Gattung *Reinekea* erwiesen sich Co-Kulturen aus zufälligen Teilpopulationen des Bakterioplanktons von Vorteil, welche scheinbar das Wachs-tum von *Reinekea* sp. in dem Medium erst ermöglichten. Diese **zufälligen Mischkulturen** wurden hergestellt indem 100 nL Aliquots des Meereswassers inokuliert wurden, in denen sich circa 50 Bakterioplanktonzellen befanden. Die Stämme der Gattungen *Polaribacter*, *Formosa*, *Gillisia*, *Reinekea* und von Verwandten der *Roseobacter* und der OM182 Gruppe hatten eine **16S rRNA Sequenzidentität von >99,8%** mit 16S rRNA Gensequenzen aus dem Bakterioplankton der Nordsee im Früh-

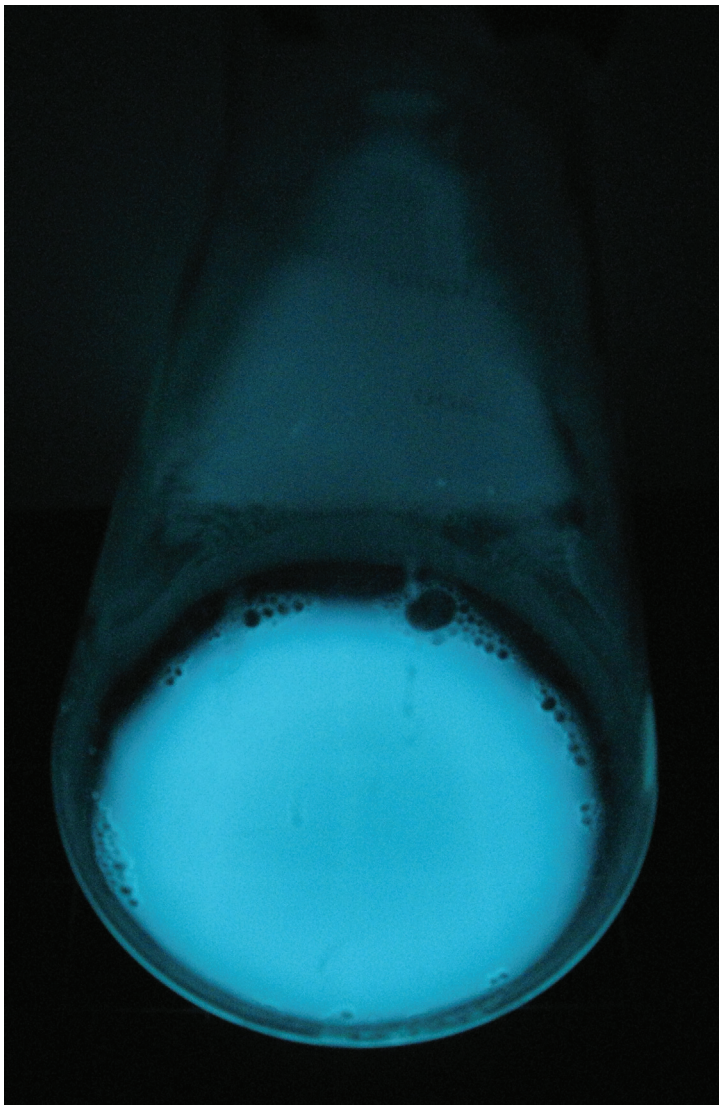
ling 2009. Der Zugang zu nahezu geschlossenen **Genomen der *Formosa*, *Polaribacter* und *Reinekea* Stämme** ermöglichte es uns, Sequenzen aus dem Bakterioplankton-Metagenom vom Frühling 2009 auf diesen Genomen abzubilden. Dabei ergab sich, dass **94% der genomischen Information** aus dem Stamm der *Formosa* Gruppe B **durch Metagenomsequenzen wiedergefunden** wurde, **90% der genomischen Information** des *Reinekea* Stammes und 50% des *Polaribacter* Stammes. Die in dieser Studie gezeigten Daten weisen darauf hin, dass **die neuen *Formosa*, *Polaribacter* und *Reinekea* Stämme repräsentative Vertreter von ökologisch bedeutsamen Bakterienarten** sind, deren Funktion vermutlich die Verstoffwechselung von Polysacchariden des von Diatomeen dominierten Phytoplanktons in der Deutschen Bucht ist.

Die physiologischen Merkmale, insbesondere das **Wachstum auf unterschiedlichen Mono- und Polysacchariden** wurde untersucht, um weitere Anhaltspunkte über die ökologischen Nischen von *Formosa*, *Polaribacter* und *Reinekea* während der Zersetzung von Algenpolysacchariden in der Nordsee zu gewinnen. Die *Polaribacter* Stämme wuchsen heterotroph auf allen getesteten sufatierten (z.B. Agar, Carrageen) und nicht-sulfatierten Polysacchariden (z.B. Zellulose, Laminarin), während die *Formosa* Stämme ausschließlich auf den nicht-sulfatierten Polysacchariden wuchsen. Im Gegensatz dazu wuchs der *Reinekea* Stamm nur auf den getesteten Monosacchariden, einschließlich N-Acetylneuraminsäure. Abschließend habe ich für diese neuen Arten die **Namen** '*Formosa flavarachnoidea*', '*Formosa forsetii*', '*Polaribacter forsetii*', '*Polaribacter frigidimaris*', '*Polaribacter adhaesivus*' und '*Reinekea forsetii*' **vorgeschlagen**.

List of abbreviations

AMP adenosine monophosphate
ANI average nucleotide identity
ASW artificial seawater
CARD-FISH catalyzed reporter deposition-fluorescence *in situ* hybridization
CAZy carbohydrate active enzymes
CBM carbohydrate binding module
CCA canonical correspondence analysis
CFU colony forming unit
CMC carboxy methyl cellulose
DAPI 4',6-diamidino-2-phenylindole
DOM dissolved organic matter
EDTA ethylenediamine-N,N,N',N'-tetraacetic acid
FISH fluorescence *in situ* hybridization
GH glycoside hydrolase
HaHa short form of the HaHa medium
HEPES 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid
HMW high molecular weight
HTC high–throughput cultivation
iTRF *in silico* terminal restriction fragment
ITS 16S–23S intergenic spacer
MALDI-TOF matrix–assisted laser desorption/ionization time of flight
nMDS nonmetric multidimensional scaling
OMG oligotrophic marine *Gammaproteobacteria*
ORF open reading frame
PCR polymerase chain reaction
PR proteorhodopsin
PSA polysialic acid
PUL polysaccharide utilization loci
RDP ribosomal database project
rRNA ribosomal RNA
Sus starch utilization system
TBDT TonB-dependent transporter
TEM transmission electron microscopy
T-RFLP terminal restriction fragment length polymorphism
TRF terminal restriction fragment
Tris-HCl Tris-(hydroxymethyl)-aminomethane
2216(E) marine 2216 medium

Contents



Bioluminescent *Aliivibrio fischeri* strain (basonym *Vibrio fischeri*) cultivated in an Erlenmeyer flask.

Chapter 1

Introduction

1.1 Cultivation based ecology

Cultivation of aerobic marine microorganisms was pioneered by Johann Friedrich Bernhard Fischer (Fig. 1.1) and Harry Luman Russel, who both used the pour-plate technique originally developed by Robert Koch (1876). During the *Plankton-Expedition der Humboldt-Stiftung* in 1989 , Bernhard Fischer (1894) developed a device for the aseptic sampling of surface seawater and deep seawater of up to 1000 meters depth. This sampling device was replaced first by the metallic Nansen bottle (Fridtjof Wedel-Jarlsberg Nansen, Nansen, 1901) and later by the plastic Niskin bottle (Shale Jack Niskin, Berube, 2005). Fischer observed the highest number of bacteria on agar plates if the agar plates were inoculated with neritic (Latin *nērītа*, 'sea mussel', shallow sea near a coastline) seawater of epeiric seas (Greek *ēpeiros*, 'continent', inland sea, e.g. Baltic Sea),

Figure 1.1 Robert Koch (left) and Bernhard Fischer (right) at the Cholera-Expedition 1883. Adapted from (Exner, 2009).

marginal seas (partially enclosed sea, e.g. North Sea), or oceanic seawater at the borders of two convergent ocean currents (Fischer, 1894). Thus, he concluded that the bacteria of the ocean lived primarily in nutrient rich habitats on the dead bodies or excretions of marine plants and animals (saprophytic).

At the same time, H. L. Russel (1891) studied benthic bacteria by sampling sediment from up to 1000 meters depth in the Gulf of Naples. On agar plates he cultivated more bacteria from surface sediments of shallow waters than of deeper waters, and more bacteria from sediment than from overlaying water. Furthermore, he observed that 35% of the morphologically distinct bacteria cultivated from the sediment were not cultivated from the overlaying seawater. Based on his findings, he proposed that the origin of the cultivated marine bacteria was mainly their natural habitat (autochthonous, *autochthon*, Greek *auto-* 'self' and *chthon* 'soil', i.e. 'sprung from earth itself'). Waksman (1934) stated that the bacterial populations of the sea are different from the ones of the soil and that the chemical composition of the habitat defines the bacterial community.

Claude Ephraim ZoBell from the Scripps Institution of Oceanography of the University of California (Fig. 1.2) had a great impact on the ecology and cultivation of aerobic marine bacteria. He developed the marine medium 2216 for the cultivation of a broad range of marine aerobic heterotrophic bacteria (ZoBell, 1941). The development of this medium is an example for the history of media components. The medium of Fischer (1894) consisted of 10 g/L meat-extract peptone and 5 g/L fish-extract peptone dissolved in natural seawater. However, ZoBell (1941) and later Buck (1974) did not observe higher bacterial numbers on the agar plate supplemented with fish-extract peptone. Standardized peptone was commercially not available at the time of Fischer. Thus, ZoBell hypothesized that the low culturability of bacteria on the agar plate was caused by the

poor quality of the self-made meat-extract peptone and the high amount in the medium of Fischer. The addition of phosphate as K_2HPO_4 and iron as ferric phosphate ($Fe(III)PO_4$) yielded more bacteria and a broader range of morphological distinct bacteria (ZoBell, 1941). Hence, the marine 2216 medium was supplemented with standardized peptone (Bacto peptone) and ferric phosphate. After studying the conditions and essential growth factors of established cultures, Koser and Saunders (1938) and Knight (1935) proposed yeast extract as growth promoting supplement. Correspondingly, the marine 2216 medium was further developed by the addition of yeast extract yielding the marine 2216E medium (Oppenheimer and ZoBell, 1952). A modification of the marine 2216E medium with basal salts (artificial seawater) instead of natural seawater is till today commercially available as BD Difco™ marine broth/agar 2216 from (BD Diagnostic Systems, USA). This medium constitutes the medium of many cultivation studies in marine microbiology, and was mentioned for the first time by Havenner and colleagues (1979). In his monograph, ZoBell (1946) reviewed the progress made in marine bacteriology since the pioneering monograph of Fischer (1894). He discussed the latitudinal-longitudinal and vertical distribution of marine bacteria and the effect of environmental factors such as temperature, season, tide, hydrostatic pressure, and phytoplankton. Furthermore, he concluded that

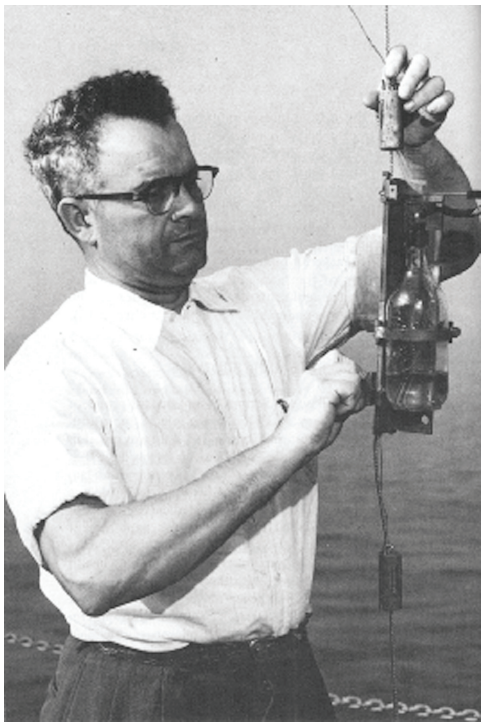


Figure 1.2 Claude E. ZoBell attaching messenger to wire above a J-Z sampler, in the 1940s. Adapted from (Scripps Institution of Oceanography, 1978).

bacteria of the genera *Pseudomonas*, *Vibrio*, *Flavobacterium*, and *Achromobacter* predominate in the ocean (ZoBell, 1946). At that time the genera *Pseudomonas* and *Vibrio* belonged to the family *Pseudomonadaceae*, the genera *Achromobacter* and *Flavobacterium* to *Achromobacteraceae*, based on the taxonomy of the first edition of the *Bergey's Manual of Deterministic Bacteriology* (Bergey et al., 1923). The studies and standardized methods of ZoBell (1946) have been a benchmark for other researchers in the field of marine ecology (McGraw, 2006; Karl and Proctor, 2007). For example, Sieburth (1967) isolated more than 2500 colonies of psychrophilic, psychrotolerant, and mesophilic bacteria on agar plates over a period of two years. He concluded on a seasonal selection of bacteria by water temperature, irrespective with which phylum they were affiliated with.

A discrepancy of microscopic counts of freshwater bacteria (direct cell counts) and the number of colonies forming on the agar plate (colony forming units, CFU), had already been reported by Bere (1933). Direct cell counts were two orders of magnitude higher than CFUs. Jannasch and Jones (1959) discussed the difficulty of counting bacteria from seawater directly by microscopy. Improved staining techniques and microscopy equipment resulted in accurate and standardized direct cell counts (Hobbie et al., 1977) and confirmed the observation that often less than 1% of marine microorganisms can be cultured on agar plates (Kogure et al., 1979). The phrase 'great plate count anomaly' was coined by Staley and Konopka (1985) to describe this phenomenon in their review of techniques to assess the autecology –the study of individual species in relation to the environment– and synecology –the study of the ecological interrelationships within communities and with their environment of microorganisms–.

1.2 Difficulties in cultivating marine bacteria

Overall, there are two major reasons for why we are not able to cultivate most of the bacteria. First, many researchers possibly overlooked putative novel bacteria (Leadbetter, 2003). For instance, oligotrophic bacteria grow to a low cell density of less than 10^6 cells per millimeter (Kuznetsov et al., 1979; Rappé et al., 2002). These low cell numbers can not be detected by measuring the optical density or the formation of colonies on the agar plate (Button et al., 1993). Hence, other techniques must be applied for the detection of bacteria in nutrient poor media, like epifluorescence microscopy (Connon and Giovannoni, 2002; Amann and Moraru, 2012) or flow cytometry (Button et al., 1993; Fuchs et al., 2000). Second, it is still impossible to recreate the nutritional requirements of many bacteria during cultivation (Leadbetter, 2003). Furthermore, bacteria are metabolically versatile and thus, cannot be cultivated in a single medium. Consequently, either filtered (Sieburth, 1967) or autoclaved (Fischer, 1894; ZoBell, 1941; Button et al., 1993) seawater was used for medium preparation. Alternatively, environmental conditions were simulated with diffusion chambers which enabled the cultivation of microorganisms in their natural habitat separated by filter discs (Kaeberlein et al., 2002).

Reasons for the inability to cultivate 99% of bacterial communities on agar plates can be linked to the factors influencing the regulation of cell metabolism or cell signaling. Environmental conditions were shown to negatively influence bacterial growth, such as low temperature, a shock of sudden nutrient abundance, osmotic pressure, reduced light, pH, antibiotics, and toxins (Postgate and Hunter, 1964; Whitesides and Oliver, 1997; Mascher, 2006; Lennon and Jones, 2011). The effect of high nutrient concentration in the medium is discussed in detail in *1.5 Concentration of organic carbon.*

Microorganisms have evolved the ability to enter a reversible state of re-

duced metabolic activity (maintenance) or of stopped growth and development (dormancy) to lower their energetic expenditures and overcome stressful conditions (Lennon and Jones, 2011). Mechanisms to enter the resting stage are activated by environmental stress (Kaprelyants et al., 1993). The mechanisms for the transition into and out of the resting stage and produced cellular structures require energy and resources (van Bodegom, 2007). Furthermore, sensory mechanism for the interpretation of favorable environmental conditions must be available for the reactivation (resuscitation) of the starved cells (Rees, 1996; Caceres and Tessier, 2003). Maintenance and dormancy must be an advantage for the microorganisms and thus have been maintained in the course of evolution. Consequently unfavorable cultivation conditions might favor dormancy.

Small signal molecules (pheromones or autoinducers) that diffuse in and out of bacterial cells are known from quorum sensing which is a cell-to-cell communication mechanism of bacterial populations to coordinate their gene expression after reaching a certain cell density (Williams et al., 2007). This exchange of information enables the bacteria to cope with environmental stress by improving their access to nutrients, generating a collective defense against other competing microorganisms, and adopting different morphologies (Williams et al., 2007). The successful cultivation of novel species through the presence of other microorganisms from the same environment was demonstrated for soil bacteria (Kaeberlein et al., 2002). D'Onofrio and colleagues (2010) showed the effect of signal molecules (e.g. siderophores) of neighboring microorganisms promoting the growth of novel bacteria. In contrast, neighboring microorganisms excreting antagonistic molecules can inhibit the growth of bacteria (Long and Azam, 2001) and colonization of surfaces (Cude et al., 2012). A cytokine-like growth factor from an active growing culture promoted the resuscitation of dormant *Micrococcus luteus* cultures (Mukamolova et al., 1998). The addition of signal molecules like

cyclic AMP (cAMP) and N-acyl homoserine lactones (AHL) increased the cultivation efficiency for seawater (Bruns et al., 2002) and freshwater bacteria (Bruns et al., 2003b).

Widdel and Pfennig (1977) observed that microaerophilic microorganisms were not able to form colonies on the surface of agar plates in an oxic environment. These microorganisms grow rather inside the solid media and can possibly be cultured by using agar shakes or agar dilution series.

1.3 Novel cultivation strategies yielding ecological relevant bacteria

In the last decades, sophisticated methods for the cultivation of novel microorganisms have been developed. The acidophilic *Thiobacillus ferrooxidans* was cultivated on floating filters, because the low pH makes an application of agar plates impossible (DeBruyn et al., 1990). A gas-lift bioreactor was used to mimic deep-sea hydrothermal ecosystems (Postec et al., 2005). The slow growing chemolitoautotrophic ammonium oxidizing *Nitrosomonas* and the nitrite oxidizing *Nitrobacter* were enriched by using a bioreactor equipped with hanging sponge-cubes (Araki et al., 1999). The isolation of slow growing microorganisms might require their separation from clumps of cells or from fast growing microbial populations. The separation of single cells with optical tweezers yielded slow growing hyperthermophilic archaea (Huber et al., 1995). Micromanipulation enabled the cultivation of novel bacterial species from the termite gut (Fröhlich and König, 2000).

Button and colleagues (1993) developed the dilution cultivation as a variation of the cultivation to extinction. They succeeded in the isolation of some representative oligotrophic marine bacteria (Schut et al., 1993). The technique and theory was named dilution cultivation and yielded the cultivation efficiency of up to 60% of the bacterial community. The procedure

was the following. The microbial community was diluted in sterile seawater near extinction, comparable to the most probable number (MPN) technique (Exworthy, 1933; Haas, 1989). However, differences were the further inoculation of the diluted bacterial communities into the medium, and the screen for growth with a low cell density (detection limit 10^3 cells per milliliter) by flow cytometry.

Dilution cultivation ultimately opened the field of high throughput cultivation. Tan and colleagues (1996) used an 8-channel pipette to speed up the preparation procedure of the dilution cultivation and compared the physiological characteristics of cultivated copiotrophic and oligotrophic bacteria from the Antarctic. Connon and Giovannoni (2002) developed a protocol for high-throughput dilution cultivation (HTC) and used fluorescence microscopy of cell arrays to detect growth in the oligotrophic medium. This technique led to the cultivation of novel species of *Proteobacteria* from marine bacterioplankton (Connon and Giovannoni, 2002), including representatives of the oligotrophic marine *Gammaproteobacteria* (OMG) group (Cho and Giovannoni, 2004). A further improvement of the medium composition was the supplementation of sterilized seawater with inorganic nitrogen and phosphorus compounds and a defined mixture of organic carbon compounds in μM concentrations. This brought the globally important *Alphaproteobacteria* clade SAR11 into culture (Rappé et al., 2002), a decade after its first discovery (Giovannoni et al., 1990). Another novel technique, the Micro-Drop microdispenser, automatically distributes droplets that received single bacterial cells into 96well plates (Bruns et al., 2003a). Moreover, microorganisms can grow in separated compartments after encapsulation single cells in gel microdroplets (Zengler et al., 2002). The bulky diffusion chambers (Kaeberlein et al., 2002) were further upgraded to the high throughput Ichip with multiple diffusion chambers (Nichols et al., 2010). On the contrary, also cultivation studies using the traditional cultivation on agar plates led to the

cultivation of novel ecological relevant bacteria. Eilers and colleagues (2001) cultivated the cosmopolitan NOR5 lineage of *Gammaproteobacteria* on synthetic seawater agar supplemented with inorganic nitrogen and phosphorus compounds in μM concentrations. The co-cultivation of marine bacteria with the axenic (bacterium-free) dinoflagellate *Lingulodinium polyedrum* in natural seawater yielded a representative strain of the *Roseobacter* NAC11-3 lineage (Mayali et al., 2008). The *Deltaproteobacteria* strain FiPS-3 revolutionized our knowledge on the phosphorus metabolism, because it was enriched and successfully isolated from an anoxic sediment by coupling the reduction of sulfate to sulfide with the oxidation of phosphite to phosphate (Schink and Friedrich, 2000). Janssen and colleagues (2002) and Sait and colleagues (2002) demonstrated the cultivation of phylogenetically diverse soil bacteria on agar plates with the polymeric substrate xylane.

1.4 Targeted cultivation based on genomic information

Metagenomics provide insights into metabolic features of so far uncultured microorganisms and in two cases this has supported cultivation. Tyson and colleagues (2005) identified the nitrogen fixation operon (*nif*) in the metagenome of an acid mine drainage sample. The *nif* genes were affiliated with the phylum *Nitrospirae* which had no cultured representative. Finally, a nitrogen free medium yielded *Leptospirillum ferrodiazotrophum* as a novel isolate from acid mine drainage. *Rikenella*-like symbionts inhabiting the digestive tract of the medicinal leech *Hirudo verbena* were successfully cultured based on the metagenomic discovery of genes encoding the degradation of sulfated and sialated mucin glycans (Bomar et al., 2011).

1.5 Biochemical considerations for the design of artificial seawater media

Elemental composition

A prokaryotic cell consists to 98% dry weight of six non-metal- (C, O, H, N, S, P) and four metal elements (K, Mg, Fe, Ca) (Overmann, 2006). The elemental composition of the seawater basal salts is quite stable in seawater of the same salinity Tab. 1.1 and Tab. 1.2. However, the available macro nutrients (C, N, S, P) or trace metals (e.g. iron, nickel) differ remarkably in quantity and quality between seasons, locations and depths. The ratio between the elements is stated different in the literature. Redfield (1934) found a molar C:N:P ratio of 106:16:1 in bacterioplankton of the surface seawater and in the seawater of the deep sea . Fleming (1940) calculated a C:N:P ratio of 105:15:1 for plankton and Sakshaug and colleagues (1983) a C:N:P ratio of 102:14:1 of nearly nutrient saturated phytoplankton communities in Norwegian seawater and freshwater. Sterner and colleagues (2008) concluded that for broad scales the Readfield ratio was consistent, but varies between habitats and species of different metabolisms (e.g. storage of polyphosphate or polyhydroxyalcanoids). Nevertheless, the amount of required phosphorus is double as high for bacteria then for algae leading to a C:N:P ratio of 50:10:1 (Fagerbakke et al., 1996) or 45:9:1 (Goldman et al., 1987). When the composition of structural components and enzymes are considered only, the sum formula of bacteria cells is $C_4H_{6.4}O_{1.5}NP_{0.09}S_{0.024}$ that equals a C:N:P ratio of 44:11:1 (Overmann, 2010).

Concentration of organic carbon

In aquatic systems heterotrophic bacteria are classified into two types of nutrient adaptation, oligotrophic and eutrophic bacteria (Kuznetsov et al.,

Table 1.1 Concentration of elements in natural seawater, exclusive of gases. ZoBell (ZoBell, 1941), Svedrup (Svedrup et al., 1942), Goldberg (Goldberg, 1965), Culkin (Culkin, 1965), Kester (Kester et al., 1967)

Element (g/kg)	ZoBell	Svedrup	Goldberg	Culkin	Kester
Chlorine	19.0	19.0	19.0	19.4	19.4
Sodium	10.5	10.6	10.5	28.0	10.8
Magnesium	1.4	1.3	1.35	1.3	1.3
Calcium	0.4	0.40	0.40	0.41	0.41
Potassium	0.38	0.38	0.38	0.39	0.39
Bromine	0.065	0.065	0.067	0.067	0.066
Strontium		0.013	0.008	0.008	0.008
Boron	0.005	0.005	0.005	0.026	0.026
Silicium	0.003	0.004	0.003		
Sulphur	0.885	0.9	8.8	2.7	2.7
Carbon	0.028	0.028	0.030		
Nitrogen	0.0005	0.0007	0.0003		
Phosphorus	0.0007	0.0001	0.00007		
Trace metal (mg/kg)					
Fluorine	13.0	1.4	1.3	1.0	1.0
Aluminium		0.5	0.01		
Iodine		0.05	0.06		
Arsenic		0.02			
Iron	0.01000	0.02	1		
Manganese		0.01			
Copper		0.01			
Zinc		0.005			
Selenium		0.004			
Molybdenum		0.0005			
Nickel		0.0001			
Salinity (‰)	34.3	34.3	35.0	35.0	35.0

1979; Giovannoni and Stingl, 2007). Eutrophic bacteria –also named copiotrophic (Poindexter, 1981) or saprophytic bacteria (Kuznetsov et al., 1979)– grow at carbon concentrations of more than 1 g/L (Yanagita et al., 1978). In contrast, oligotrophic bacteria are able to grow at carbon concentrations of 1–15 mg/L, but not at higher carbon concentrations (Kuznetsov et al., 1979; Ishida and Kadota, 1981; Button et al., 1993). For example,

the *Alphaproteobacteria* clade SAR11 and the *Gammaproteobacteria* OMG (oligotrophic marine *Gammaproteobacteria*) group are common in the oligotrophic oceans (Giovannoni et al., 1990; Cho and Giovannoni, 2004) that have organic carbon concentrations between 30–200 μM, corresponding to 0.36–2.4 mg/L organic carbon (Jannasch et al., 1996). Representatives of both the SAR11 clade and the OMG group, as well as *Sphingomonas alaskensis* (*Alphaproteobacteria*) are obligate oligotrophic bacteria that can grow only under oligotrophic conditions and to a cell density of less than 10^6 cells per milliliter (Schut et al., 1997; Connon and Giovannoni, 2002; Rappé et al., 2002). Facultative oligotrophic bacteria are able to grow at both low and high carbon concentrations (Schut et al., 1993; Ishida et al., 1982). In cultivation experiments many research groups observed a significantly reduced amount of cultivable freshwater and seawater bacteria when the medium was supplemented with high concentrations ($>$ 1 g/L) of nutrient broth, peptone or yeast extract (Buck, 1974; Martin and MacLeod, 1984; Schut et al., 1993; Jensen et al., 1996; Bussmann et al., 2001; Janssen et al., 2002). This suggests that oligotrophic bacteria are more abundant in aquatic environments than copiotrophic bacteria.

Most of the marine microorganisms that are available in culture collections were isolated with high nutrient concentrations of more than one gram per liter. These fast growing microorganisms are overrepresented in culture collections (Keller and Zengler, 2004). Typical exaples of theses copiotrophs are the genera *Vibrio*, *Alteromonas*, and *Pseudoalteromonas*. These are often isolated from seawater, but accounted most often for less than one percent of the total bacterial community of seawater (Eilers et al., 2000; Pedrós-Alió, 2006). This culture-induced enrichment of low abundant or rare bacterial populations (Stevens et al., 2009) was often described when nutrient-enriched media were used in batch cultures with synthetic seawater (Eilers et al., 2000) or filtered seawater (Fuchs et al., 2000) and chemostats

Table 1.2 Composition of synthetic seawater, exclusive of organic components. 1940, (Lyman and Fleming, 1940); 2216, BD DIFCO™ 2216; 1992,(Widdel and Bak, 1992); 1993,(Schut et al., 1993); 1996,(Atlas, 1996); 2009a,(Stevens et al., 2009); 2009b,(Winkelmann and Harder, 2009); 2012,(Carini et al., 2012). *, expressed as kg^{-1}

Component	1940*	2216	1992	1993	1996	2009a	2009b	2012
Base salts (g/L)								
NaCl	23.5	19.5	20	30	28	18	26.37	28.11
$MgCI_2$ $6H_2O$	5.0	8.8	3.0	1.0	2.6	11.4	5.67	5.49
Na_2SO_4	3.9	3.2	4.0	4.0				
$MgSO_4$ $7H_2O$					4.0		6.8	0.69
$CaCI_2$ $6H_2O$	1.1	1.8	0.15	0.15	1.2	1.5	1.47	1.47
KCl	0.66	0.55	0.5	0.7	0.8	0.7	0.72	0.67
KBr	0.10	0.08		0.1		0.09	0.1	
Macronutrients (mg/L)								
NH_4Cl				270		400		
NH_4NO_4			1.6					
$(NH_4)_2SO_4$								5300
KH_2PO_4	2.1	8.0	204	270			500	
NaH_2PO_4								600
Trace metal (mg/L)								
$SrCl_2$	24	34		40			20	
H_3BO_3	27	22	0.03	25		0.03	20	
Na_4O_4Si		4.0						
NaF	3.0	2.0		1.0			3.0	
$FeSO_4$ $7H_2O$			2.1				2.1	
$FeCl_3$ $6H_2O$				2		2.1		0.032
Trace metal (μg/L)								
$MnCl_2$ $4H_2O$			100	80		100	100	1.8
$CuCl_2$ $2H_2O$			2	5		2	2	
ZnSO4 $7H_2O$			144	60		144	144	0.23
Na_2SeO_3 $5H_2O$			6	15		6	6	0.17
Na_2MoO_4 $2H_2O$			36	75		36	36	0.1
$NiCl_2$ $6H_2O$			24	20		24	24	0.24
$CoCl_2$ $6H_2O$			190	5		190	190	0.12

with filtered seawater (Massana and Jürgens, 2003). Hence, high nutrient concentrations in the medium might inhibit the growth of most marine bacteria (Olsen and Bakken, 1987), because they are not adapted to high amounts of carbon in the medium (Bussmann et al., 2001). Consequently, the amount of carbon in the medium selects for bacteria.

Trace elements

The bioavailability of the transition metals such as iron, manganese, cobalt, nickel, copper, zinc, molybdenum, and tungsten is low in aquatic, non-acidic and oxygenated environments. In contrast to alkali (Na^+, K^+) and alkali earth metals (Mg^{2+}, Ca^{2+}), transition metals are soft Lewis acids with a high binding stability to soft Lewis bases (e.g. sulfur as FeS, Fe_2S) in aqueous environments. This leads to the precipitation of transition metals as oxides or hydroxides under these conditions (Argüello et al., 2012).

Transition metals are required as prosthetic groups of metalloproteins for many essential physiological processes in the cell mediating electron transfer and redox reactions (Overmann, 2006; Andreini et al., 2008; Dupont et al., 2010). However, at high concentrations transition metals catalyze the production of free radicals or substitute for other metal cofactors (Argüello et al., 2012). During earth history abundances of transition metals changed from iron and manganese dominated environments to copper and zinc dominated environments and thereby influenced the bioavailability of these elements as cofactors for biochemical processes (Dupont et al., 2010). Furthermore, bacteria have developed alternative strategies to get access to transition metals at low concentration or complexed with other molecules. For example bacteria are able to acquire iron with siderophores, free heme or the heme-containing proteins hemophores, lactoferrin and transferin (Sandy and Butler, 2009). Thus, for our cultivation, the medium was supplemented with a trace element solution to which ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) was added as chelator (Widdel and Bak, 1992).

Buffer and pH

Many buffers are available for the cultivation of bacteria at a pH between 7.0 and 8.0, the pH range at which most of the marine bacteria were cul-

tivated (Fig. 1.3) by ZoBell (ZoBell, 1941, 1946). In earlier times, most often Tris-(hydroxymethyl)-aminomethane (short Tris-HCl) or phosphate buffer were used to control the pH (Good et al., 1966). Tris (hydroxymethyl) aminomethane has a low buffering capacity below a pH of 7.5 and might be inhibitory due to the primary amino group (Good et al., 1966). Such inhibition was shown for α-amylase (Ghalanbor et al., 2008) and aminopeptidase (Desmarais et al., 2002). The inorganic phosphate buffer was shown to inhibit the growth of bacteria from low nutrient environments, known as substrate-accelerated death (Postgate and Hunter, 1964). Furthermore, concentrations of phosphate in the mM range led to the formation of precipitates with trace elements (e.g. Fe^{3+}) and bivalent cations such as Mg^{2+} and Ca^{2+} (Bartscht et al., 1999; Overmann, 2006). Good and colleagues (1966) synthesized hydrogen ion buffers (Good's buffers) which were inert to chemical or biological degradation and had improved buffer capacities. Among these buffers, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (HEPES) and 3-(N-morpholino) propanesulfonic acid (MOPS) both had a pK_a at 7.5 and did not form complexes with metal ions. The comparison of the phosphate buffer with HEPES and MOPS revealed a significant lower culturability for cultures buffered with phosphate buffer and highest numbers in cultivation were achieved with HEPES

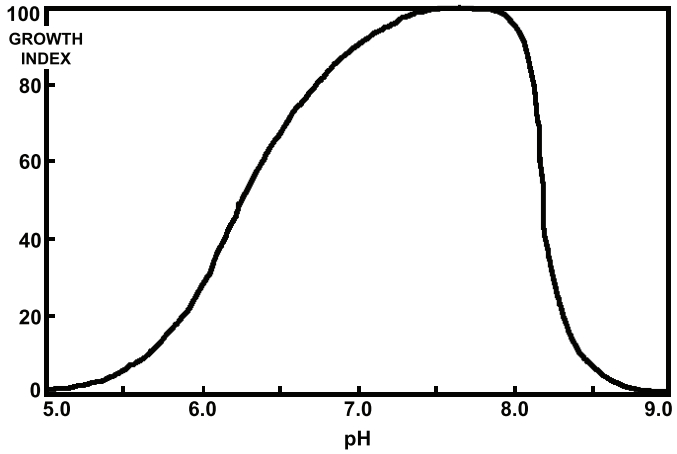


Figure 1.3 Relative amount of colony forming units (growth index) at a certain pH observed on nutrient rich marine agar 2216. Adapted from (ZoBell, 1941, 1946).

(Bartscht et al., 1999). For copper susceptible species such as the marine dinoflagellate *Amphidinium carterae* HEPES can enhance copper toxicity by increasing the bioavailability of copper(II) (Lage et al., 1996; Vasconcelos et al., 1996).

Bicarbonate is the natural buffer in seawater and is in equilibrium with the CO_2 in the atmosphere at a concentration of 2 mM (ZoBell, 1941). However, at this low concentration the bicarbonate buffer is not sufficient to maintain a stable pH during prolonged incubation in nutrient rich medium (ZoBell, 1946; Bartscht et al., 1999). Thus, Widdel and Bak (1992) applied a concentration of 30 mM bicarbonate, but only in closed gas tight bottles.

Pressure

Molecular systems are not effected by elevated pressures of up to 1,013 kPa (Follonier et al., 2012) and mesophilic bacteria are able to grow at pressures of up to 30 MPa (ZoBell and Johnson, 1949). Moreover, marine bacteria are able to withstand higher pressures of up to 50 MPa (ZoBell and Johnson, 1949). Nevertheless, pressure can effect the concentrations of dissolved gases (e.g. oxygen, carbon dioxide) and thus affects bacterial metabolism indirectly (Follonier et al., 2012). The effects of high pressure on bacteria are summarized by Follonier and colleagues (2012). In this study, all samples were surface waters from Sylt, Janssand, Harlesiel, and Helgoland and therefore pressure effects were no issue.

1.6 The family of *Flavobacteriaceae*

Flavobacteriaceae inhabit a huge variety of environments in the biosphere (Kirchman, 2002; Bernardet and Nakagawa, 2006). They form important populations of heterotrophic bacteria (Bernardet and Nakagawa, 2006) in soils (Johansen and Binnerup, 2002; Johansen et al., 2009), freshwater

(Jaspers et al., 2001; Kirchman, 2002), marine (Eilers et al., 2000; Kirchman, 2002; Alonso et al., 2007; Teeling et al., 2012), and industrial environments (Whiteley and Bailey, 2000). Members of the *Flavobacteriaceae* were also found in extreme habitats such as the surface of the deep-sea (Schauer et al., 2010), hypersaline solar saltern ponds (Baati et al., 2008), and polar regions (Bowman et al., 1997; Ravenschlag et al., 2001; Gómez-Pereira et al., 2010) including permanently cold sediments of the Arctic (Ravenschlag et al., 2001) and Antarctic (Bowman et al., 2003).

The taxonomy of *Flavobacteriaceae* before 2006

The family of *Flavobacteriaceae* was first suggested in the PhD thesis of Jooste (1985) and mentioned in the first edition of the *Bergey's Manual of Systematic Bacteriology* (Reichenbach, 1989). Almost a decade later, the validation and description of the family *Flavobacteriaceae* (Bernardet et al., 1996) and the minimal standards for the description of new taxa of the family were published (Bernardet et al., 2002). This family belongs to the phylum *Bacteroidetes* (Krieg et al., 2010) which was formerly known as the 'Flavobacter-Bacteroides' phylum (Gherna and Woese, 1992), the *Cytophaga/Flavobacterium/Bacteroides* line (Hirsch et al., 1998) or the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group (Weller et al., 2000). Members of the *Flavobacteriaceae* are heterotrophic, gram-negative bacteria, with various morphologies, from coccoid or short rods to long filaments (Bernardet, 2010). In the last version of *Bergey's Manual of Systematic Bacteriology* (editorial deadline June 2006) the family of *Flavobacteriaceae* comprised 168 species in 53 genera (Bernardet, 2010). Type strains originating from marine habitats (29 genera with 67 species) dominated the free-living (10 genera with 13 species) as well as the free-living/saprophytic *Flavobacteriaceae* (25 genera with 67 species) (Bernardet, 2010). These

included two of the oldest genera, *Flavobacterium* and *Chryseobacterium*, which comprised 54 species of diverse life-styles (e.g. free-living, parasitic or saprophytic) isolated from terrestrial, freshwater, and marine environments (Bernardet, 2010).

Taxonomic changes in the family of *Flavobacteriaceae* since 2006

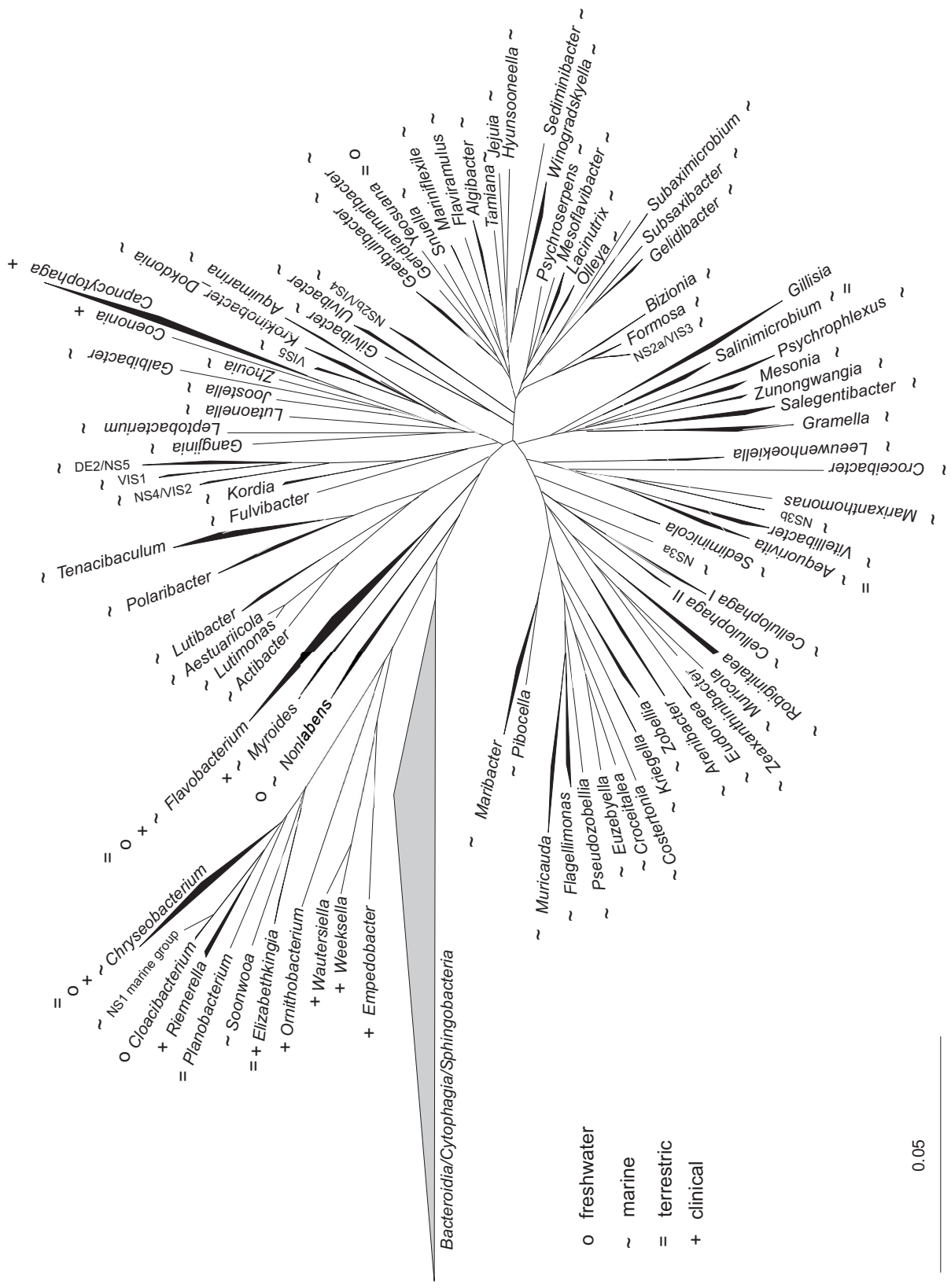
Since 1997, the *List of Prokaryotic names with Standing in Nomenclature*, formerly known as the *List of Bacterial names with Standing in Nomenclature* (LBSN) follows the description and reclassification of bacteria with their official names as cited in the *Approved Lists of Bacterial Names* and validly published in the *International Journal of Systematic and Evolutionary Microbiology* formerly the *International Journal of Systematic Bacteriology*. This list is available at http://www.bacterio.cict.fr (Euzéby, 1997) and it was used as the basis of the following update of *Flavobacteriaceae* nomenclature.

Since the editorial deadline June 2006 of the last version of *Bergey's Manual of Systematic Bacteriology* to July 2012 the family *Flavobacteriaceae* has expanded significantly. Descriptions of 49 new genera and 226 new species were added and 40 species and several genera were reclassified. The genus *Donghaeana* was reclassified as *Persicivirga* (Nedashkovskaya et al., 2009), *Kaistella* and *Sejongia* as *Chryseobacterium* (Kämpfer et al., 2009a,b), *Stanierella* and *Gaetbulimicrobium* as *Aquimarina* (Nedashkovskaya et al., 2006), *Persicivirga*, *Stenothermobacter* and *Sandarakinotalea* as *Nonlabens* (Yi and Chun, 2012). In June 2012, the family *Flavobacteriaceae* consisted of 393 type strains in 95 genera (Fig. 1.4). The minor amount of 9 genera with 19 type strains were either of clinical origin (15 species), associated to dying animals (3 species) or their wounds (1 species). Furthermore, the genus *Planobacterium* was of terrestrial origin and the genera

Epilithonimonas and *Cloacibacterium* were from freshwater environments, solely. Still, the oldest genera *Flavobacterium* and *Chryseobacterium* were the largest genera comprising 146 species originating from clinical, terrestrial, freshwater and marine environments (Tab. 2.S2 in Chapter 2 at page 87). Only, seven of these species were isolated from seawater and marine sediment. Nevertheless, 80 *Flavobacteriaceae* genera with 210 type strains are of marine origin, clearly demonstrating their high diversity in this habitat (Fig. 2.S1 in Chapter 2 at page 103). Pigmentation, gliding motility, iridescence and decomposition of complex organic material are impressive characteristics of *Flavobacteriaceae*.

Pigmentation

The name *Flavobacteriaceae* originated from *flavus* (L. adj. masc. *flavus* yellow), reporting the light to bright yellow or even orange colony color (Bernardet et al., 2002; Bernardet, 2010) of type strains in 90 genera (Tab. 2.S2 in Chapter 2). The colony color is based on carotenoid-type pigments (identified in 70 genera), flexirubin-type pigments (identified in 10 genera), and undescribed pigments (in 10 genera) (Bernardet and Nakagawa, 2006) (Tab. 2.S2 in Chapter 2). Flexirubins were more frequently observed in *Flavobacteriaceae* of clinical, freshwater or terrestrial origin, while marine *Flavobacteriaceae* often had carotenoids (Reichenbach et al., 1980; Bernardet, 2010). Flexirubin-type pigments were reported from the marine *Flavobacteriaceae* genera *Aquimarina* (9 species), *Kriegella* (1), *Nonlabens* (1), *Pseudozobellia* (1), *Ulvibacter* (2), *Vitellibacter* (2), and *Zobellia* (5) (Tab. 2.S2 in Chapter 2). In contrast to the localization of carotenoids in the inner cytoplasmic membrane, flexirubin-type pigments are located in the outer membrane of *Cytophagia*, *Flavobacteria* and *Sphingobacteria* (Irschik and Reichenbach, 1978; Bernardet and Nakagawa, 2006).


Bacteroidia/Cytophagia/Sphingobacteria
Empedobacter
Weeksella
Wautersiella
Ornithobacterium
Elizabethkingia
Soonwooa
Planobacterium
Riemerella
Cloacibacterium
NS1 marine group
Chryseobacterium
Myroides
Nonlabens
Flavobacterium
Actibacter
Lutimonas
Aestuariicola
Lutibacter
Polaribacter
Tenacibacterium
Fulvibacter
Kordia
NS4/VIS2
VIS1
DE2/NS5
Gangjinia
Leptobacterium
Lutaonella
Joostella
Zhouia
Galbibacter
Ulvibacter
Krokinobacter
Aquimarina
Dokdonia
Capnocytophaga
Coenonia
VIS5
NS2b/VIS4
Gaetbulibacter
Gentianimonas
Yeosuana
Snuella
Mariniflexile
Flaviramulus
Algibacter
Tamiana
Jejuia
Hyunsooneella
Sediminibacter
Winogradskyella
Psychroserpens
Mesoflavibacter
Lacinutrix
Olleya
Subaximicrobium
Subsaxibacter
Gelidibacter
Bizionia
Formosa
NS2a/VIS3
Gillisia
Salinimicrobium
Psychrophlexus
Mesonia
Zunongwangia
Salegentibacter
Gramella
Leeuwenhoekiella
Croceibacter
Marixanthomonas
NS3b
Vitellibacter
Aequorivita
NS3a
Sediminicola
Cellulophaga I
Cellulophaga II
Robiginitalea
Zeaxanthinibacter
Muricola
Eudoraea
Arenibacter
Zobellia
Krieglella
Costertonia
Croceitalea
Euzebyella
Pseudozobellia
Flagellimonas
Muricauda
Pibocella
Maribacter
o freshwater
~ marine
= terrestric
+ clinical
0.05

Flexirubin-type pigments are identified by a simple assay: upon addition of 20% KOH flexirubins change their color from yellow/orange to red/purple/brown. This can be reversed by acid addition (Reichenbach et al., 1980). This assay is not specific for flexirubin-type pigments (Reichenbach et al., 1980), but phenolic carotenoids (e.g. of *Brevibacterium linens*) change their color from yellow-orange to pink-purple (Kohl et al., 1983) and KOH test positive xanthomonadins were found in the genus *Xanthomonas* (Andrewes et al., 1973). Furthermore, a KOH treatment without an observed bathochromic shift might be the result of a modification of the phenolic hydroxyl group (Fautz and Reichenbach, 1980). Thus, for a further species description of questionable strains the pigments should be extracted and analyzed (Bernardet et al., 2002).

The function of flexirubin-type pigments is unknown, but the biosynthesis of flexirubin proceeds only in growing cells (Fautz and Reichenbach, 1980). In contrast, carotenoids pigments promote light harvesting (Clayton 1953) and phototaxis (Thomas and Goedheer, 1953), protect against photodynamic killing (Mathews and Sistrom, 1959) by removing oxygen radicals (Blass et al., 1959).

Figure 1.4 ***(facing page)*** Phylogenetic relationship among type strains and lineages without cultured representatives of the family *Flavobacteriaceae*. The phylogenetic tree is based on comparisons of 16S rRNA gene sequences using the neighbour-joining method and a 0% and 40% base frequency filter of *Bacteroidetes*. Type strains of the classes *Bacteroidia*, *Cytophagia* and *Sphingobacteria* were used as outgroups. The isolation source is indicated by: o, freshwater; ∼, marine environment; = terrestrial environment; +, clinical samples. *Flavobacteria* clades which had so far no representative culture are indicated by VIS (Gómez-Pereira et al., 2010), NS (Alonso et al., 2007) and DE (Kirchman et al., 2003). Scalebar represents 5 nucleotide substitutions per 100 nucleotides.

Gliding motility

Flavobacteria are non-motile or move by gliding (Bernardet, 2010). It was proposed that an attachment to surfaces of phytoplankton and algae or the colonization of biofilms would help to stay close to the substratum (Reichenbach, 1981; Gómez-Pereira et al., 2010). Bacteria that are able to move by gliding have different advantages, (i) movement is possible on a rather dry surface, (ii) penetration into and migration within a complex organic matrix enables the bacteria to reach their polymeric substrates that do not diffuse, (iii) and movement in a fluidic environment is possible without loosing the contact to the substratum (Reichenbach, 1981). Gliding motility on surfaces such as solid agar or algae is mediated by diverse mechanisms and have a higher calcium requirement (Burchard, 1980; Overmann, 2006), as shown for *Desulfonema magnum* (Widdel et al., 1983).

Since, flagella or pili are absent in members of the class *Flavobacteria* (Bernardet, 2010) swimming in aqueous environments or swarming across solid surfaces does not occur (Jarrell et al., 2008). Furthermore, an ATP driven twitching motility with the type IV pili is known for species of *Proteobacteria*, *Cyanobacteria* and gram-positive bacteria (Jarrell et al., 2008), but the energy for movement of *Flavobacteria* is provided by proton motive force (McBride, 2001). Jarrell and McBride (2008) discussed the model of polysaccharide extrusion (known for *Myxococcus*), derived from the association of *Flavobacteria* to polysaccharide attachment and degradation. However, this model is unlikely, because (i) latex spheres of nanometer scale did not move directed near the bacteria cell surface (Nelson et al., 2008), (ii) and a rapid movement of *Flexibacter* sp. BH3 was observed even without exogenous nutrients and with an emptied carbon storage (McBride, 2001).

Lateral movement of cell surface adhesins can mediate gliding motility, as shown for *Flavobacterium johnsoniae* (Nelson et al., 2008). Motor proteins

are anchored to the peptidoglycan and move adhesins which are attached to the substratum. These motor proteins are driven by proton motive force (Jarrell et al., 2008). Furthermore, among 36 *Flavobacteriaceae* genera with strains with described gliding motility, 34 genera were of marine origin (Tab. 2.S2 in Chapter 2). This suggests that gliding motility is important for *Flavobacteria* in marine environments.

Iridescence

Iridescence is the colored appearance of an object depending on the angle of direct illumination caused by architectures in the nanometer-scale (Vukusic and Sambles, 2003; Doucet and Meadows, 2009; Meadows et al., 2009). It has been described from colonies of *Flavobacteria* (Bernardet, 2010; Kientz et al., 2012a). However, iridescence is not part of the minimal standards for describing new taxa of the family *Flavobacteriaceae* (Bernardet et al., 2002). ZoBell (1946) described iridescence as greenish fluorescence which was found among 7% of agar plate cultures of marine origin. Recently, Kientz and colleagues (2012a) developed methods for the standardized determination of iridescence by trans- and epi-illumination. Furthermore, this group defined for the first time categories of iridescence. A coupling of iridescence with the gliding motility was hypothesized for the establishment of the iridescent structures (Kientz et al., 2012b). During their studies of abiotic factors that influence iridescence in *Cellulophaga lytica* (*Flavobacteriaceae*) it was shown that iridescence occurred under cold and water stress (Kientz et al., 2012b). Furthermore, the physical structures that cause iridescence might support thermoregulation, UV protection, filtering of light, water repellence, reduced mechanical friction and prevention of desiccation, for bacteria in the marine environment (Doucet and Meadows, 2009).

The marine clade of *Flavobacteriaceae*

The chemoheterotrophic *Flavobacteriaceae* are a major component of the bacterioplankton in aquatic ecosystems and are often found associated with phytoplanktonic primary production (Kirchman, 2002). Thus, the marine clade of *Flavobacteriaceae* was proposed which consisted of 19 genera of exclusively marine origin (Bowman, 2006). Since its emended description in 2006, 55 new genera of *Flavobacteriaceae* have been described exclusively from marine habitats (Fig. 1.4). Bowman (2006) hypothesized on an evolutionary expansion of *Flavobacteriaceae* from marine habitats to non marine environments. The motivation was based on a clear distinct phylogenetic cluster of the marine clade of *Flavobacteriaceae* (e.g. *Maribacter*, *Aquimarina*) and species in genera that were found exclusively in terrestrial and freshwater environments (e.g. *Chryseobacterium*, *Planobacterium*, *Epilithonimonas*, *Cloacibacterium*). The ecological transition state was represented by genera inhabiting a wide range of terrestrial and aquatic environments (e.g. *Flavobacterium*, *Salinimicrobium*).

Flavobacteriaceae in the marine environments

The marine realm can be split in a benthic and a pelagic zone. The benthic zone is an ecological region that includes the sediment surface and sub-surface layers such as shores, underwater rocks, corals, and intertidal sediment. The pelagic zone is the water column that goes from the bottom of the sea to the sea surface including oceanic, and coastal waters. In marine environments most of the *Bacteroidetes* can be phylogenetically affiliated with the class *Flavobacteria*, mainly *Flavobacteriaceae* (Kirchman, 2002; Alonso et al., 2007; Teeling et al., 2012). Abundances of this bacteria group have been obtained by fluorescence *in situ* hybridization (FISH) with the probes CF319a (Manz et al., 1996) and CFB560 (O'Sullivan et al., 2002).

Both probes have a different coverage in the *Bacteroidetes* as reviewed by Amann and Fuchs (2008) and Diez-Vives and colleagues (2012).

Highest abundances of *Flavobacteria* were found in nutrient rich (eutrophic) ecosystems, suggesting a preference for these habitats (Kirchman, 2002; Gómez-Pereira et al., 2010). The bacterial communities in an nutrient rich upwelling area consisted of significant more *Flavobacteria* than in the surrounding seawater, with up to 30% and 10% respectively (Alonso-Sáez et al., 2007, 2012). In the photic zone 50% of the net primary production (photosynthesis) is remineralized by the heterotrophic bacteria community (Azam, 1998) that consists of up to 20% *Flavobacteria* (Schattenhofer et al., 2009; Gómez-Pereira et al., 2010). Particles in the ocean are hotspots of organic matter (Azam and Long, 2001) and are significantly colonized by *Flavobacteria*, like the particle-associated fraction of the picoplankton (Simon et al., 1999; Abell and Bowman, 2005; Gómez-Pereira et al., 2010), formed particles of the estuarine turbidity maxima (Crump et al., 1999), and marine snow (Woebken et al., 2007). *Flavobacteria* are of significant abundance accounting for 15% to 25% of the bacteria community in the intertidal sediment of the North Sea (Llobet-Brossa et al., 1998; Musat et al., 2006). During phytoplankton blooms, *Flavobacteria* are of high abundance irrespective of the season. In summer *Flavobacteria* accounted for 30% of total cell counts in the coastal surface seawater (Eilers et al., 2000). Moreover, *Flavobacteria* accounted for up to 25% in the austral summer in the Scotia Arc (Jamieson et al., 2012) and for 70% during a *Phaeocystis* sp. bloom (Simon et al., 1999). A comparable *Flavobacteria* abundance of 60% of the bacteria community was observed during a bloom of *Phaeocystis* sp. and *Thalassiosira* sp. in spring 2009 (Teeling et al., 2012). Thus, *Flavobacteria* were of highest abundance in nutrient rich (eutrophic) ecosystems, suggesting a preference for these habitats (Kirchman, 2002; Gómez-Pereira et al., 2010).

However, there is evidence that different *Flavobacteria* lineages occupy different ecological niches in relation to the available algal primary products (Kirchman, 2002; Gómez-Pereira et al., 2010; Teeling et al., 2012). For example, Riemann and colleagues (2000) and Pinhassi and colleagues (2004) showed that a shift in the phytoplankton community composition from phytoflagellates to diatoms resulted in distinct *Flavobacteria* phylotypes. West and colleagues (2008) could show the difference in dominating *Flavobacteria* lineages within and outside of the phytoplankton bloom. Moreover, during the decomposition of the spring phytoplankton bloom in the German Bight a successive occurrence of different *Flavobacteria* clades was observed (Teeling et al., 2012).

Decomposition of complex organic matter

Waksman and colleagues (1933) proposed that bacteria are responsible for the decomposition and further remineralization of complex organic matter. *Flavobacteria* participate in the initial degradation of complex organic matter (Edwards et al., 2010; Gómez-Pereira et al., 2010; Thomas et al., 2011; Teeling et al., 2012) and profit first from a decaying phytoplankton bloom (Teeling et al., 2012). Hence, they are responsible for a major fraction of organic matter remineralization in the oceans (Kirchman, 2002).

Besides amino acids, polysaccharides are a major fraction of organic matter in the ocean (Benner et al., 1992; Dittmar et al., 2001; Koch et al., 2005) and their initial breakdown with extracellular enzymes is the rate limiting step (Arnosti, 2003, 2010). Complex polysaccharides are composed of different carbohydrate moieties linked by diverse glycosidic bonds. Their degradation requires a set of synergistic acting glycoside hydrolases (Warren, 1996). A successive occurrence of hydrolases was shown for the bacterioplankton community in mesocosms experiments (Riemann et al., 2000).

Teeling and colleagues (2012) followed the succession of bacterial populations that are involved in the decomposition of the spring phytoplankton bloom in the German Bight by a combination of cultivation-independent methods. In the early phase, *Formosa* sp. dominated the *Bacteroidetes* mainly expressing glycoside hydrolases for the decomposition of non-sulfated laminarin, whereas in the late phase *Polaribacter* sp. dominated the production of sulfatases for the decomposition of more complex sulfated carbohydrates (Teeling et al., 2012).

Indeed, *Bacteroidetes* consume rather polymeric organic matter (e.g. chitin, proteins) than amino acids, in contrast to *Alphaproteobacteria* and *Gammaproteobacteria* (Cottrell and Kirchman, 2000). Furthermore, *Bacteroidetes* have evolved a novel machinery to utilize polysaccharides (Shipman et al., 2000; Xu et al., 2003) whose components are located mostly in the periplasm and outer membrane or are secreted into the medium (Luo, 2012). This machinery (Fig. 1.5) was first described as starch utilization system (Sus) in *Bacteroides thetaiotaomicron* and consisted of at least the two outer membrane bound proteins SusC and SusD, and glycoside hydrolases at the outer membrane and in the periplasm (Shipman et al., 2000). Homologous proteins of SusD bind specifically to oligomeric carbohydrates and deliver them to proteins homologous to SusC. SusC-like proteins are TonB-dependent transporters that transport oligosaccharides via a beta-barrel through the outer membrane (McBride et al., 2009; Martens et al., 2011). This transport of molecules larger than 600 kDa requires the interaction with an inner membrane protein complex consisting of TonB, ExbB, and ExbD, which itself derives the energy from the proton motive force (Noinaj et al., 2010). A sensor-regulator system controls the expression of downstream genes which either consists of an extra-cytoplasmic function sigma(ECF-sigma)/anti-sigma factor pairs or a hybrid two-component system (HTCS). This regulation system acts as sensor for oligosaccharides at

the outer membrane and thus does not require an uptake of the oligosaccharides into the periplasm (Koebnik, 2005).

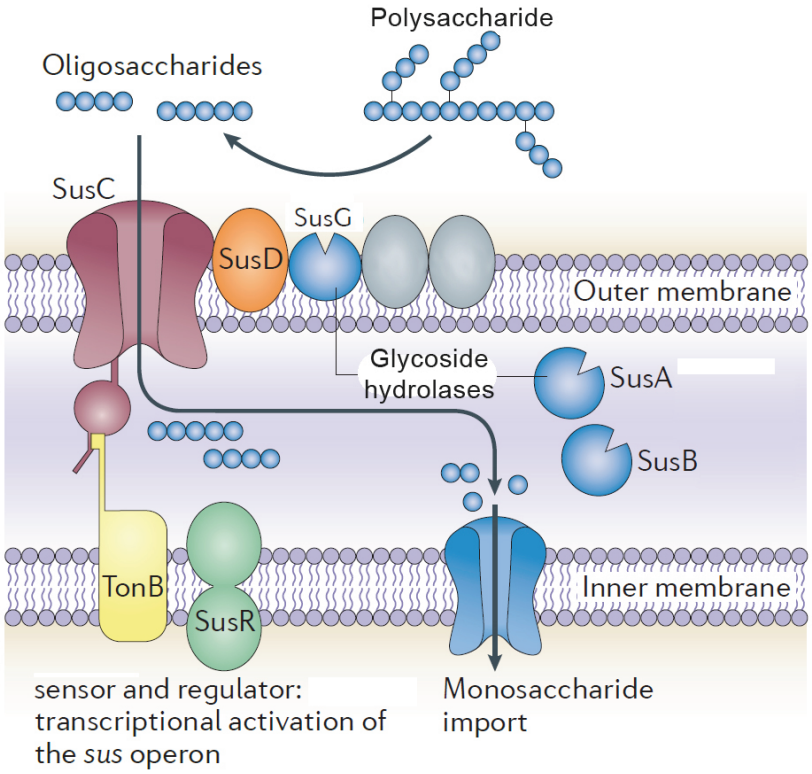


Figure 1.5 The machinery for polymer decomposition, first described as starch utilization system (Sus), contains SusD proteins for the specific substrate binding, and glycoside hydrolases at the outer membrane to decompose the polysaccharides into oligosaccharides. The oligosaccharides are transported into the periplasm by an ATP driven TonB-dependent transporter and are further decomposed by periplasmic glycoside hydrolases into monosaccharides. Adapted from Koropatkin et al. (2012)

Genes encoding this machinery, including carbohydrate active enzymes (CAZy), are often found to be co-localized in polysaccharide utilization loci (PUL) which are activated by defined oligosaccharides (Cantarel et al., 2009; Martens et al., 2011). For example, the genome of the marine polysaccharide-degrading '*Gramella forsetii*' (class *Flavobacteria*) encoded for 40 glycoside hydrolases (10.5 per Mbp, genome 3.8 Mbp) of which more than half of them were localized in the direct vicinity of TonB-dependent transporters (Bauer et al., 2006). The 6.1 Mbp large genome of the chiti-

nolytic soil bacterium *Flavobacterium johnsoniae* (Stanier, 1947; Bernardet et al., 1996) encoded for 138 glycoside hydrolases and 9 polysaccharide lyases, 42 TonB-dependent transporters and 29 sigma factors (McBride et al., 2009). In fosmids of the North Atlantic Ocean a high frequency of TonB-dependent transporters (9 per Mbp) and PULs were found (Gómez-Pereira et al., 2012). The others suggested that they originate from *Flavobacteria* involved in the degradation of phytoplankton derived sulfated polysaccharides. A metaproteomic study in the South Atlantic identified 19% of the proteins as TonB-dependent transporters and most of them were closely related to those found in *Bacteroidetes* in coastal samples (Morris et al., 2010). Based on the finding that both TonB-dependent transporters and rhodopsins were identified in the same lineages the authors proposed a beneficial effect of light on transport activities (Morris et al., 2010). The role of TonB-dependent transporter was also pronounced during the bacterial decomposition of the spring phytoplankton bloom in the North Sea. The proportion of TonB-dependent transporter in the expressed proteins almost doubled from 7% before the bloom to 13% during the algae decomposition and was dominated by *Flavobacteria* (Teeling et al., 2012). Furthermore, different *Flavobacteria* clades showed distinct profiles of carbohydrate active enzymes and transporter, suggesting an occupation of different ecological niches provided by algal primary products (Teeling et al., 2012). *Flavobacteria* may have developed a specific substrate utilization machinery that enables oligomer uptake as soon as carbohydrates become available. This trait possibly enabled them to succeed in specific ecological niches within zones of high net primary production (Teeling et al., 2012).

1.7 The North Sea

The German Bight in the North Sea is a shallow coastal area with high tidal dynamics (Port et al., 2011). Its sandy seafloor is a major sink of organic matter and nitrogen species originating mainly from terrestrial areas, estuarine discharge of freshwater, and from open sea (Alongi, 1998; Gao et al., 2012). The Wadden Sea in the German Bight has a coastline of 500 km and encompasses an area of 14,000 km^2. Its seafloor represents the largest tidal system in the world in which 93% of the seafloor is dominated by coarse, sandy, or mixed sediments (Reise et al., 2010). Moreover one-third of the Wadden Sea area (4,700 km^2) is exposed to tidal changes (Reise et al., 2010). The German Bight is a rectangular basin with dominating eastwards winds forming an anticlockwise (cyclonic) wind current and tide along the shore line (Port et al., 2011). Counteracting discharges of eutrophic freshwater, mainly from the rivers Elbe and Weser (1000 $m^3\ s^{-1}$) almost stop the penetration of water from the open ocean (Port et al., 2011). Furthermore, the resulting stratification turns the direction of the incoming eastwards flow to north-northwest. Thus, ocean processes in the German Bight are mostly driven by tides where shallow coastal areas of less than 20 meter depth are strongly exposed to tidal mixing (Port et al., 2011). Differences in the microbial community of intertidal sediments are linked to the organic matter content (Llobet-Brossa et al., 1998), as well as the organic material and microbial communities of sedimenting aggregates (Novitsky, 1990). Hence, heterotrophic bacteria capable to decompose organic matter play an important role in the coastal area of the North Sea.

1.8 Aims and Objectives

In the epipelagic zone of the ocean, aerobic heterotrophic bacteria remineralize half of the photosynthetically produced organic material. The remineralization is mainly driven by members of the classes *Alphaproteobacteria*, *Gammaproteobacteria*, and *Flavobacteria*. The specific function of these microorganisms in their habitat is unclear and representative strains in culture are rare. Therefore, the aim of this thesis is the **isolation of *Flavobacteria* and the isolation of *in situ* abundant marine bacteria by improving cultivation media and procedures**.

Flavobacteria are common in coastal waters and benthic habitats. Previous studies showed significant abundances of *Flavobacteria* in the seawater and the sediment of the North Sea. However, cultivation approaches obtained a low number of *Flavobacteria* isolates, irrespective whether they originated from seawater, intertidal sediment or algae. The first step was the design of **a new artificial seawater medium** for the cultivation of phylogenetically diverse marine *Flavobacteriaceae*. It was important that this medium excludes fast growing opportunistic bacteria. **Different benthic and pelagic environmental samples** were investigated and different cultivation techniques on agar plates were applied to increase the diversity of *Flavobacteriaceae* isolates. These phylogenetically diverse isolates exhibited a broad range of colony and cell morphology, and often inconspicuous morphological characteristics. The second step therefore was the design of **a specific PCR assay for the identification of *Flavobacteriaceae***.

Even though this strain collection will be diverse, it may follow the 'great plate count anomaly' and thus, the obtained isolates might not reflect the *Flavobacteriaceae* taxa that were identified in bacterial metagenomes and clone libraries of the North Sea. Several bacterioplankton populations of the three classes *Flavobacteria*, *Alphaproteobacteria*, and *Gammaproteobacteria*

have recently been shown to benefit from the decomposition of the spring phytoplankton bloom in 2009. However, representative strains of these bacterioplankton populations were not in culture. Therefore, **an artificial seawater medium of environmental-like nutrient concentrations** was developed and combined with **dilution cultivation**. Field work at Helgoland, a fast processing of the seawater and incubation near *in situ* temperature were considered to **maximize the culturability**. Flow cytometry was applied to **detect growth in the medium at low cell densities**. For the **taxonomic affiliation** of obtained isolates, 16S rRNA gene sequences were compared with full-length 16S rRNA gene clones of bacterioplankton of the 2009 spring bloom. Furthermore, **draft genomes** of selected isolates were used to **recruit reads of metagenomes from bacterioplankton** of the 2009 spring bloom and thus, **addressing the ecological relevance of the isolates in the North Sea**.

Among the taxonomic affiliation of the isolates, **physiological characteristics** of selected isolates were investigated to deepen the knowledge of niche differentiation during phytoplankton decomposition. The focus was on **mono- and polysaccharides utilization**, substrate requirements and morphological characteristics.

References

Abell, G. C. J. and Bowman, J. P. (2005). Colonization and community dynamics of class *Flavobacteria* on diatom detritus in experimental mesocosms based on Southern Ocean seawater. *FEMS Microbiol Ecol* **53**, 379–391.

Alongi, D. (1998). *Coastal ecosystem processes*. CRC Press, Boca Raton, USA.

Alonso, C., Warnecke, F., Amann, R. and Pernthaler, J. (2007). High local and global diversity of *Flavobacteria* in marine plankton. *Environ Microbiol* **9**, 1253–1266.

Alonso-Sáez, L., Arístegui, J., Pinhassi, J., Gómez-Consarnau, L., González, J. M., Vaqué, D., Agustí, S. and Gasol, J. M. (2007). Bacterial assemblage structure and carbon metabolism along a productivity gradient in the NE Atlantic Ocean. *Aquat Microb Ecol* **46**, 43–53.

Alonso-Sáez, L., Sánchez, O. and Gasol, J. M. (2012). Bacterial uptake of low molecular weight organics in the subtropical Atlantic: Are major phylogenetic groups functionally different? *Limnol Oceanogr* **57**, 798–808.

Amann, R. and Fuchs, B. M. (2008). Single-cell identification in microbial communities by improved fluorescence *in situ* hybridization techniques. *Nat Rev Microbiol* **6**, 339–348.

Amann, R. and Moraru, C. (2012). Two decades of fluorescence *in situ* hybridization in systematic and applied microbiology. *Syst Appl Microbiol* **35**, 483–484.

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L. and Thornton, J. M. (2008). Metal ions in biological catalysis: from enzyme databases to general principles. *J Biol Inorg Chem* **13**, 1205–1218.

Andrewes, A. G., Hertzber, S., Liaaenje, S. and Starr, M. P. (1973). The *Xanthomonas* 'carotenoids' - noncarotenoid, brominated, aryl-polyene esters. *Acta Chem Scand* **27**, 2383–2395.

Araki, N., Ohashi, A., Machdar, I. and Harada, H. (1999). Behaviors of nitrifiers in a novel biofilm reactor employing hanging sponge-cubes as attachment site. *Water Sci Technol* **39**, 23–31.

Argüello, J. M., Raimunda, D. and González-Guerrero, M. (2012). Metal transport across biomembranes: emerging models for a distinct chemistry. *J Biol Chem* **287**, 13510–13517.

Arnosti, C. (2003). Fluorescent derivatization of polysaccharides and carbohydrate-containing biopolymers for measurement of enzyme activities in complex media. *J Chromatogr B: Anal Technol Biomed Life Sci* **793**, 181–191.

Arnosti, C. (2010). Microbial extracellular enzymes and the marine carbon cycle. *Annu Rev Mar Sci* **3**, 401–425.

Atlas, R. M. (1996). *Handbook of microbiological media 2nd Edn*, chapter ASW Medium. Parker L. (ed), p. 1545. CRC Press, New York.

Azam, F. (1998). Microbial control of oceanic carbon flux: The plot thickens. *Science* **280**, 694–696.

Azam, F. and Long, R. A. (2001). Oceanography – Sea snow microcosms. *Nature* **414**, 495–498.

Baati, H., Guermazi, S., Amdouni, R., Gharsallah, N., Sghir, A. and Ammar, E. (2008). Prokaryotic diversity of a Tunisian multipond solar saltern. *Extremophiles* **12**, 505–518.

Bartscht, K., Cypionka, H. and Overmann, J. (1999). Evaluation of cell activity and of methods for the cultivation of bacteria from a natural lake community. *FEMS Microbiol Ecol* **28**, 249–259.

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H., Knaust, F. et al. (2006). Whole genome analysis of the marine bacteroidetes '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**, 2201–2213.

Benner, R., Pakulski, J. D., McCarthy, M., Hedges, J. I. and Hatcher, P. G. (1992). Bulk chemical characteristics of dissolved organic matter in the ocean. *Science* **255**, 1561–1564.

Bere, R. (1933). Numbers of bacteria in inland lakes of wisconsin as shown by the direct microscopic method. *Int Rev Hydobiolo* **29**, 248–263.

Bergey, D. H., Harrison, F. C., Breed, R. S., Hammer, B. W. and Huntoon, F. M. (1923). *Bergey's Manual of Deterministic Bacteriology.* Williams and Wilkins, Baltimore, MD, USA.

Bernardet, J.-F. (2010). *Bergey's Manual of Systematic Bacteriology. The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes Vol 4*, chapter Class II. *Flavobacteriia* class. nov. Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., Paster, B.J., Ward, N.L. et al. (eds), pp. 106–314. Springer, New York.

Bernardet, J. F., Nakagawa, Y., and Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049–1070.

Bernardet, J.-F. and Nakagawa, Y. (2006). *The Prokaryotes*, chapter An Introduction to the family *Flavobacteriaceae*. Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., Stackebrandt E., Bernardet J.-F., Nakagawa Y. (eds), pp. 455–480. Springer, New York.

Bernardet, J. F., Segers, P., Vancanneyt, M., Berthe, F., Kersters, K. and Vandamme, P. (1996). Cutting a gordian knot: emended classification and description of the genus *Flavobacterium*, emended description of the family *Flavobacteriaceae*, and proposal of *Flavobacterium hydatis* nom. nov. (basonym, *Cytophaga aquatilis* Strohl and Tait 1978). *Int J Syst Bacteriol* **46**, 128–148.

Berube, M. S. (2005). *The American Heritage scince dictionary*, chapter Nansen bottle. Pickett, J.P. and Leonesio, C. and Spitz, S. (eds), p. 421. Houghton Mifflin Company, Boston, MA, USA.

Blass, U., Anderson, J. M. and Calvin, M. (1959). Biosynthesis and possible functional relationships among the carotenoids: and between chlorophyll *a* and chlorophyll *b*. *Plant Physiol* **34**, 329–333.

Bomar, L., Maltz, M., Colston, S. and Graf, J. (2011). Directed culturing of microorganisms using metatranscriptomics. *mBio* **2**, e00012–11.

Bowman, J. P. (2006). *The Prokaryotes*, chapter The marine clade of the family *Flavobacteriaceae*: The genera *Aequorivita*, *Arenibacter*, *Cellulophaga*, *Croceibacter*, *Formosa*, *Gelidibacter*, *Gillisia*, *Maribacter*, *Mesonia*, *Muricauda*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Robigini-*

talea, *Salegentibacter*, *Tenacibaculum*, *Ulvibacter*, *Vitellibacter* and *Zobellia*. Bowman, J.P., Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., and Stackebrandt, E. (eds), pp. 677–694. Springer, New York.

Bowman, J. P., McCammon, S. A., Brown, M. V., Nichols, D. S. and McMeekin, T. A. (1997). Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* **63**, 3068–3078.

Bowman, J. P., McCammon, S. A., Gibson, J. A. E., Robertson, L. and Nichols, P. D. (2003). Prokaryotic metabolic activity and community structure in Antarctic continental shelf sediments. *Appl Environ Microbiol* **69**, 2448–2462.

Bruns, A., Cypionka, H. and Overmann, J. (2002). Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. *Appl Environ Microbiol* **68**, 3978–3987.

Bruns, A., Hoffelner, H. and Overmann, J. (2003a). A novel approach for high throughput cultivation assays and the isolation of planktonic bacteria. *FEMS Microbiol Ecol* **45**, 161–171.

Bruns, A., Nübel, U., Cypionka, H. and Overmann, J. (2003b). Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. *Appl Environ Microbiol* **69**, 1980–1989.

Buck, J. D. (1974). Effects of medium composition on recovery of bacteria from sea water. *J Exp Mar Biol Ecol* **15**, 25–34.

Burchard, R. P. (1980). Gliding motility of bacteria. *Bioscience* **30**, 157–162.

Bussmann, I., Philipp, B. and Schink, B. (2001). Factors influencing the cultivability of lake water bacteria. *J Microbiol Methods* **47**, 41–50.

Button, D. K., Schut, F., Quang, P., Martin, R. and Robertson, B. R. (1993). Viability and isolation of marine bacteria by dilution culture - theory, procedures, and initial results. *Appl Environ Microbiol* **59**, 881–891.

Caceres, C. E. and Tessier, A. J. (2003). How long to rest: the ecology of optimal dormancy and environmental constraint. *Ecology* **84**, 1189–1198.

Cantarel, B., Coutinho, P., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009). The Carbohydrate-Active enZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* **37**, D233–D238.

Carini, P., Steindler, L., Beszteri, S. and Giovannoni, S. J. (2012). Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. *ISME J* **7**, 592–602.

Cho, J. C. and Giovannoni, S. J. (2004). Cultivation and growth characteristics of a diverse group of oligotrophic marine *Gammaproteobacteria*. *Appl Environ Microbiol* **70**, 432–440.

Connon, S. A. and Giovannoni, S. J. (2002). High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol* **68**, 3878–3885.

Cottrell, M. T. and Kirchman, D. L. (2000). Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster

consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66**, 1692–1697.

Crump, B. C., Armbrust, E. V. and Baross, J. A. (1999). Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. *Appl Environ Microbiol* **65**, 3192–3204.

Cude, W. N., Mooney, J., Tavanaei, A. A., Hadden, M. K., Frank, A. M., Gulvik, C. A., May, A. L. and Buchan, A. (2012). Production of the antimicrobial secondary metabolite indigoidine contributes to competitive surface colonization by the marine roseobacter *Phaeobacter* sp. strain Y4I. *Appl Environ Microbiol* **78**, 4771–4780.

Culkin, F. (1965). *Chemical Oceanography Vol 1*, chapter The major constituents of sea water. Riley J.P., Skirrow G. (eds), pp. 121–161. Academic Press, London.

DeBruyn, J. C., Boogerd, F. C., Bos, P. and Kuenen, J. G. (1990). Floating filters, a novel technique for isolation and enumeration of fastidious, acidophilic, iron-oxidizing, autotrophic bacteria. *Appl Environ Microbiol* **56**, 2891–2894.

Desmarais, W. T., Bienvenue, D. L., Bzymek, K. P., Holz, R. C., Petsko, G. A. and Ringe, D. (2002). The 1.20 Å resolution crystal structure of the aminopeptidase from *Aeromonas proteolytica* complexed with Tris: a tale of buffer inhibition. *Structure* **10**, 1063–1072.

Diez-Vives, C., Gasol, J. M. and Acinas, S. G. (2012). Evaluation of marine *Bacteroidetes*-specific primers for microbial diversity and dynamics studies. *Microb Ecol* **64**, 1047–1055.

Dittmar, T., Fitznar, H. P. and Kattner, G. (2001). Origin and biogeochemical cycling of organic nitrogen in the eastern Arctic Ocean as evident from D- and L-amino acids. *Geochim Cosmochim Acta* **65**, 4103–4114.

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., Clardy, J. and Lewis, K. (2010). Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem Biol* **17**, 254–264.

Doucet, S. M. and Meadows, M. G. (2009). Iridescence: a functional perspective. *J R Soc Interface* **6**, S115–S132.

Dupont, C. L., Butcher, A., Valas, R. E., Bourne, P. E. and Caetano-Anollés, G. (2010). History of biological metal utilization inferred through phylogenomic analysis of protein structures. *Proc Natl Acad Sci USA* **107**, 10567–10572.

Edwards, J. L., Smith, D. L., Connolly, J., McDonald, J. E., Cox, M. J., Joint, I., Edwards, C. and McCarthy, A. J. (2010). Identification of carbohydrate metabolism genes in the metagenome of a marine biofilm community shown to be dominated by *Gammaproteobacteria* and *Bacteroidetes*. *Genes* **1**, 371–384.

Eilers, H., Pernthaler, J., Glöckner, F. O. and Amann, R. (2000). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66**, 3044–3051.

Eilers, H., Pernthaler, J., Peplies, J., Glöckner, F. O., Gerdts, G. and Amann, R. (2001). Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. *Appl Environ Microbiol* **67**, 5134–5142.

Euzéby, J. P. (1997). List of bacterial names with standing in nomenclature: A folder available on the Internet. *Int J Syst Bacteriol* **47**, 590–592.

Exner, M. (2009). Die Entdeckung der Cholera-Ätiologie durch Robert Koch 1883/84. *Institut für Hygiene und Öffentliche Gesundheit der Universität* **Bonn, Germany**.

Exworthy, A. (1933). *American Journal of Public Health and the Nations Health, Vol 23*, chapter Standard methods for the examination of water and sewage, 7th Edn, pp. 645–646. Amer Public Health Assoc Inc, Washington.

Fagerbakke, K. M., Heldal, M. and Norland, S. (1996). Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. *Aquat Microb Ecol* **10**, 15–27.

Fautz, E. and Reichenbach, H. (1980). A simple test for flexirubin-type pigments. *FEMS Microbiol Lett* **8**, 87–91.

Fischer, B. (1894). *Die Bakterien des Meeres nach den Untersuchungen der Plankton-Expedition: unter gleichzeitiger Berücksichtigung einiger älterer und neuerer Untersuchungen.* Lipsius and Tischer, Kiel, Germany.

Fleming, R. H. (1940). Composition of plankton and units for reporting populations and production. *Proceedings 6th Pacific Science Congress 3* pp. 535–540.

Follonier, S., Panke, S. and Zinn, M. (2012). Pressure to kill or pressure to boost: a review on the various effects and applications of hydrostatic pressure in bacterial biotechnology. *Appl Microbiol Biotechnol* **93**, 1805–1815.

Fröhlich, J. and König, H. (2000). New techniques for isolation of single prokaryotic cells. *FEMS Microbiol Rev* **24**, 567–572.

Fuchs, B. M., Zubkov, M. V., Sahm, K., Burkill, P. H. and Amann, R. (2000). Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. *Environ Microbiol* **2**, 191–201.

Gao, H., Matyka, M., Liu, B., Khalili, A., Kostka, J. E., Collins, G., Jansen, S., Holtappels, M., Jensen, M. M., Badewien, T. H. et al. (2012). Intensive and extensive nitrogen loss from intertidal permeable sediments of the Wadden Sea. *Limnol Oceanogr* **57**, 185–198.

Ghalanbor, Z., Ghaemi, N., Marashi, S. A., Amanlou, M., Habibi-Rezaei, M., Khajeh, K. and Ranjbar, B. (2008). Binding of Tris to *Bacillus licheniformis* alpha-amylase can affect its starch hydrolysis activity. *Protein Pept Lett* **15**, 212–214.

Gherna, R. and Woese, C. (1992). A partial phylogenetic analysis of the 'Flavobacter-Bacteroides' phylum: basis for taxonomic restructuring. *Syst Appl Microbiol* **15**, 513–521.

Giovannoni, S. and Stingl, U. (2007). The importance of culturing bacterioplankton in the 'omics' age. *Nat Rev Microbiol* **5**, 820–826.

Giovannoni, S. J., Britschgi, T. B., Moyer, C. L. and Field, K. G. (1990). Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**, 60–63.

Goldberg, E. D. (1965). *Chemical Oceanography Vol 1*, chapter Minor elements in sea water. Riley JP, Skirrow G (eds), pp. 163–196. Academic Press, New York.

Goldman, J. C., Caron, D. A. and Dennett, M. R. (1987). Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. *Limnol Oceanogr* **32**, 1239–1252.

Gómez-Pereira, P. R., Fuchs, B. M., Alonso, C., Oliver, M. J., van Beusekom, J. E. E. and Amann, R. (2010). Distinct flavobacterial communities in contrasting water masses of the North Atlantic Ocean. *ISME J* **4**, 472–487.

Gómez-Pereira, P. R., Schüler, M., Fuchs, B. M., Bennke, C., Teeling, H., Waldmann, J., Richter, M., Barbe, V., Bataille, E., Glöckner, F. O. et al. (2012). Genomic content of uncultured *Bacteroidetes* from contrasting oceanic provinces in the North Atlantic Ocean. *Environ Microbiol* **14**, 52–66.

Good, N. E., Winget, G. D., Winter, W., Connolly, T. N., Izawa, S. and Singh, R. M. M. (1966). Hydrogen ion buffers for biological research. *Biochemistry* **5**, 467–477.

Haas, C. N. (1989). Estimation of microbial densities from dilution count experiments. *Appl Environ Microbiol* **55**, 1934–1942.

Havenner, J. A., McCardell, B. A. and Weiner, R. M. (1979). Development of defined, ninimal, and complete media for the growth of *Hyphomicrobium neptunium. Appl Environ Microbiol* **38**, 18–23.

Hirsch, P., Ludwig, W., Hethke, C., Sittig, M., Hoffmann, B. and Gallikowski, C. A. (1998). *Hymenobacter roseosalivarius* gen. nov., sp. nov. from continental Antarctic soils and sandstone: bacteria of the *Cytophaga/Flavobacterium/Bacteroides* line of phylogenetic descent. *Syst Appl Microbiol* **21**, 374–383.

Hobbie, J. E., Daley, R. J. and Jasper, S. (1977). Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl Environ Microbiol* **33**, 1225–1228.

Huber, R., Burggraf, S., Mayer, T., Barns, S. M., Rossnagel, P. and Stetter, K. O. (1995). Isolation of a hyperthermophilic archaeum predicted by *in situ* RNA analysis. *Nature* **376**, 57–58.

Irschik, H. and Reichenbach, H. (1978). Intracellular location of flexirubins in *Flexibacter elegans* (*Cytophagales*). *Bioch Biophys Acta* **510**, 1–10.

Ishida, Y., Imai, I., Miyagaki, T. and Kadota, H. (1982). Growth and uptake kinetics of a facultatively oligotrophic bacterium at low nutrient concentrations. *Microb Ecol* **8**, 23–32.

Ishida, Y. and Kadota, H. (1981). Growth patterns and substrate requirements of naturally occurring obligate oligotrophs. *Microb Ecol* **7**, 123–130.

Jamieson, R. E., Rogers, A. D., Billett, D. S. M., Smale, D. A. and Pearce, D. A. (2012). Patterns of marine bacterioplankton biodiversity in the surface waters of the Scotia Arc, Southern Ocean. *FEMS Microbiol Ecol* **80**, 452–468.

Jannasch, H. W. and Jones, G. E. (1959). Bacterial populations in sea water as determined by different methods of enumeration. *Limnol Oceanogr* **4**, 128–139.

Jannasch, H. W., Wirsen, C. O. and Doherty, K. W. (1996). A pressurized chemostat for the study of marine barophilic and oligotrophic bacteria. *Appl Environ Microbiol* **62**, 1593–1596.

Janssen, P. H., Yates, P. S., Grinton, B. E., Taylor, P. M. and Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. *Appl Environ Microbiol* **68**, 2391–2396.

Jarrell, K. F., and McBride, M. J. (2008). The surprisingly diverse ways that prokaryotes move. *Nat Rev Micro* **6**, 466–476.

Jaspers, E., Nauhaus, K., Cypionka, H. and Overmann, J. (2001). Multitude and temporal variability of ecological niches as indicated by the diversity of cultivated bacterioplankton. *FEMS Microbiol Ecol* **36**, 153–164.

Jensen, P. R., Kauffman, C. A. and Fenical, W. (1996). High recovery of culturable bacteria from the surfaces of marine algae. *Mar Biol* **126**, 1–7.

Johansen, J. E. and Binnerup, S. J. (2002). Contribution of *Cytophaga*-like bacteria to the potential of turnover of carbon, nitrogen, and phosphorus by bacteria in the rhizosphere of barley (*Hordeum vulgare* L.). *Microb Ecol* **43**, 298–306.

Johansen, J. E., Nielsen, P. and Binnerup, S. J. (2009). Identification and potential enzyme capacity of flavobacteria isolated from the rhizosphere of barley (*Hordeum vulgare* L.). *Can J Microbiol* **55**, 234–241.

Jooste, P. (1985). *The taxonomy and significance of Flavobacterium–Cytophaga strains from dairy sources*. Ph.D. thesis, University of the Orange Free State, Bloemfontein, South Africa.

Kaeberlein, T., Lewis, K. and Epstein, S. S. (2002). Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. *Science* **296**, 1127–1129.

Kämpfer, P., Lodders, N., Vaneechoutte, M. and Wauters, G. (2009a). Transfer of *Sejongia antarctica*, *Sejongia jeonii* and *Sejongia marina* to the genus *Chryseobacterium* as *Chryseobacterium antarcticum*

comb. nov., *Chryseobacterium jeonii* comb. nov. and *Chryseobacterium marinum* comb. nov. *Int J Syst Evol Microbiol* **59**, 2238–2240.

Kämpfer, P., Vaneechoutte, M., Lodders, N., De Baere, T., Avesani, V., Janssens, M., Busse, H.-J. and Wauters, G. (2009b). Description of *Chryseobacterium anthropi* sp. nov. to accommodate clinical isolates biochemically similar to *Kaistella koreensis* and *Chryseobacterium haifense*, proposal to reclassify *Kaistella koreensis* as *Chryseobacterium koreense* comb. nov. and emended description of the genus *Chryseobacterium*. *Int J Syst Evol Microbiol* **59**, 2421–2428.

Kaprelyants, A. S., Gottschal, J. C. and Kell, D. B. (1993). Dormancy in non-sporulating bacteria. *FEMS Microbiol Rev* **104**, 271–286.

Karl, D. M. and Proctor, L. M. (2007). Foundations of microbial oceanography. *Oceanography* **20**, 16–27.

Keller, M. and Zengler, K. (2004). Tapping into microbial diversity. *Nat Rev Microbiol* **2**, 141–150.

Kester, D. R., Duedall, I. W., Connors, D. N. and Pytkowicz, R. M. (1967). Preparation of artificial seawater. *Limnol Oceanogr* **12**, 176–179.

Kientz, B., Marie, P. and Rosenfeld, E. (2012a). Effect of abiotic factors on the unique glitter-like iridescence of *Cellulophaga lytica*. *FEMS Microbiol Lett* **333**, 101–108.

Kientz, B., Vukusic, P., Luke, S. and Rosenfeld, E. (2012b). Iridescence of a marine bacterium and classification of prokaryotic structural colors. *Appl Environ Microbiol* **78**, 2092–2099.

Kirchman, D. L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.

Kirchman, D. L., Yu, L. Y. and Cottrell, M. T. (2003). Diversity and abundance of uncultured *Cytophaga*-like bacteria in the Delaware Estuary. *Appl Environ Microbiol* **69**, 6587–6596.

Knight, B. (1935). An essential growth factor for *Staphylococcus aureus*. *Br J Exp Pathol* **16**, 315–326.

Koch, B. P., Witt, M. R., Engbrodt, R., Dittmar, T. and Kattner, G. (2005). Molecular formulae of marine and terrigenous dissolved organic matter detected by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. *Geochim Cosmochim Acta* **69**, 3299–3308.

Koch, R. (1876). Die Ätiologie der Milzbrand-Krankheit, begründet auf die Entwicklungsgeschichte des *Bacillus anthracis*. *Cohns Beitrage zur Biologie der Pflanzen 2* pp. 277–310.

Koebnik, R. (2005). TonB-dependent trans-envelope signalling: the exception or the rule? *Trends Microbiol* **13**, 343–347.

Kogure, K., Simidu, U. and Taga, N. (1979). A tendative direct microscopic method for counting living marine bacteria. *Can J Microbiol* **25**, 415–420.

Kohl, W., Achenbach, H. and Reichenbach, H. (1983). The pigments of *Brevibacterium linens*: aromatic carotenoids. *Phytochemistry* **22**, 207–210.

Koropatkin, N. M., Cameron, E. A. and Martens, E. C. (2012). How glycan metabolism shapes the human gut microbiota. *Nat Rev Microbiol* **10**, 323–335.

Koser, S. A. and Saunders, F. (1938). Accessory growth factors for bacteria and related microorganisms. *Bacteriol Rev* **2**, 99–160.

Krieg, N. R., Ludwig, W., Euzéby, J. and Whitman, W. (2010). *Bergey's Manual of Systematic Bacteriology: The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes, Vol 4, 2nd Edn*, chapter Phylum XIV. *Bacteroidetes* phyl. nov. Krieg N., Staley J.T., Brown D.R., Hedlund B.P., Paster B.J., Ward N.L., Ludwig W. and Whitman W.B. (eds), pp. 25–469. Springer, New York.

Kuznetsov, S. I., Dubinina, G. A. and Lapteva, N. A. (1979). Biology of oligotrophic bacteria. *Annu Rev Microbiol* **33**, 377–387.

Lage, O. M., Vasconcelos, M., Soares, H., Osswald, J. M., Sansonetty, F., Parente, A. M. and Salema, R. (1996). Suitability of the pH buffers 3-[*N-N-bis*(hydroxethyl)amino]-2-hydroxypropanesulfonic acid and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid for *in vitro* copper toxicity studies. *Arch Environ Contam Toxicol* **31**, 199–205.

Leadbetter, J. R. (2003). Cultivation of recalcitrant microbes: cells are alive, well and revealing their secrets in the 21st century laboratory. *Curr Opin Microbiol* **6**, 274–281.

Lennon, J. T. and Jones, S. E. (2011). Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat Rev Microbiol* **9**, 119–130.

Llobet-Brossa, E., Rosselló-Móra, R. and Amann, R. (1998). Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Appl Environ Microbiol* **64**, 2691–2696.

Long, R. A. and Azam, F. (2001). Antagonistic interactions among marine pelagic bacteria. *Appl Environ Microbiol* **67**, 4975–4983.

Luo, H. W. (2012). Predicted protein subcellular localization in dominant surface ocean bacterioplankton. *Appl Environ Microbiol* **78**, 6550–6557.

Lyman, J. and Fleming, R. H. (1940). Composition of sea water. *J Mar Res* **3**, 134–146.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. and Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* **142**, 1097–1106.

Martens, E. C., Lowe, E. C., Chiang, H., Pudlo, N. A., Wu, M., McNulty, N. P., Abbott, D. W., Henrissat, B., Gilbert, H. J., Bolam, D. N. et al. (2011). Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol* **9**, e1001221.

Martin, P. and MacLeod, R. A. (1984). Observations on the distinction between oligotrophic and eutrophic marine bacteria. *Appl Environ Microbiol* **47**, 1017–1022.

Mascher, T. (2006). Intramembrane-sensing histidine kinases: a new family of cell envelope stress sensors in *Firmicutes* bacteria. *FEMS Microbiol Lett* **264**, 133–144.

Massana, R. and Jürgens, K. (2003). Composition and population dynamics of planktonic bacteria and bacterivorous flagellates in seawater chemostat cultures. *Aquat Microb Ecol* **32**, 11–22.

Mathews, M. M. and Sistrom, W. R. (1959). Function of carotenoid pigments in non-photosynthetic bacteria. *Nature* **184**, 1892–1893.

Mayali, X., Franks, P. J. S. and Azarn, F. (2008). Cultivation and

ecosystem role of a marine *Roseobacter* clade-affiliated cluster bacterium. *Appl Environ Microbiol* **74**, 2595–2603.

McBride, M. J. (2001). Bacterial gliding motility: multiple mechanisms for cell movement over surfaces. *Annu Rev Microbiol* **55**, 49–75.

McBride, M. J., Xie, G., Martens, E. C., Lapidus, A., Henrissat, B., Rhodes, R. G., Goltsman, E., Wang, W., Xu, J., Hunnicutt, D. W. et al. (2009). Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. *Appl Environ Microbiol* **75**, 6864–6875.

McGraw, D. J. (2006). The founding of modern marine microbiology: Claude ZoBell and his magnum opus, 1946. *J Oceanogr* **18**, 2–13.

Meadows, M. G., Butler, M. W., Morehouse, N. I., Taylor, L. A., Toomey, M. B., McGraw, K. J. and Rutowski, R. L. (2009). Iridescence: views from many angles. *J R Soc Interface* **6**, S107–S113.

Morris, R. M., Nunn, B. L., Frazar, C., Goodlett, D. R., Ting, Y. S. and Rocap, G. (2010). Comparative metaproteomics reveals ocean-scale shifts in microbial nutrient utilization and energy transduction. *ISME J* **4**, 673–685.

Mukamolova, G. V., Yanopolskaya, N. D., Kell, D. B. and Kaprelyants, A. S. (1998). On resuscitation from the dormant state of *Micrococcus luteus*. *Anton Leeuw Int J G* **73**, 237–243.

Musat, N., Werner, U., Knittel, K., Kolb, S., Dodenhof, T., van Beusekom, J. E. E., de Beer, D., Dubilier, N. and Amann, R. (2006). Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea. *Syst Appl Microbiol* **29**, 333–348.

Nansen, F. (1901). Some oceanographical results of the expedition with the 'Michael Sars' in the Summer of 1900. Preliminary Report. *Nyt Mag Naturvidensk* **39**, Christiania.

Nedashkovskaya, O. I., Kwon, K. K. and Kim, S. J. (2009). Reclassification of *Donghaeana dokdonensis* Yoon et al. 2006 as *Persicivirga dokdonensis* comb. nov. and emended descriptions of the genus *Persicivirga* and of *Persicivirga xylanidelens* O'Sullivan et al. 2006. *Int J Syst Evol Microbiol* **59**, 824–827.

Nedashkovskaya, O. I., Vancanneyt, M., Christiaens, L., Kalinovskaya, N. I., Mikhailov, V. V. and Swings, J. (2006). *Aquimarina intermedia* sp. nov., reclassification of *Stanierella latercula* (Lewin 1969) as *Aquimarina latercula* comb. nov. and *Gaetbulimicrobium brevivitae* Yoon et al. 2006 as *Aquimarina brevivitae* comb. nov. and emended description of the genus *Aquimarina*. *Int J Syst Evol Microbiol* **56**, 2037–2041.

Nelson, S. S., Bollampalli, S. and McBride, M. J. (2008). Sprb is a cell surface component of the *Flavobacterium johnsoniae* gliding motility machinery. *J Bacteriol* **190**, 2851–2857.

Nichols, D., Cahoon, N., Trakhtenberg, E. M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K. and Epstein, S. S. (2010). Use of Ichip for high-throughput *in situ* cultivation of "uncultivable" microbial species. *Appl Environ Microbiol* **76**, 2445–2450.

Noinaj, N., Guillier, M., Barnard, T. J. and Buchanan, S. K. (2010). TonB-dependent transporters: regulation, structure, and function. *Annu Rev Microbiol* **64**, 43–60.

Novitsky, J. (1990). Evidence for sedimenting particles as the origin of

the microbial community in a coastal marine sediment. *Mar Ecol Prog Ser* **60**, 161–167.

Olsen, R. A. and Bakken, L. R. (1987). Viability of soil bacteria: optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. *Microb Ecol* **13**, 59–74.

Oppenheimer, C. H. and ZoBell, C. E. (1952). The growth and viability of 63 species of marine bacteria as influenced by hydrostatic pressure. *J Mar Res* **11**, 10–18.

O'Sullivan, L. A., Weightman, A. J. and Fry, J. C. (2002). New degenerate *Cytophaga-Flexibacter-Bacteroides*-specific 16S ribosomal DNA-targeted oligonucleotide probes reveal high bacterial diversity in River Taff epilithon. *Appl Environ Microbiol* **68**, 201–210.

Overmann, J. . (2006). *The Prokaryotes Vol 1*, chapter Principles of enrichment, isolation, cultivation and preservation of prokaryotes. Dworkin M., Falkow S., Rosenberg E., Schleifer K.-H., Stackebrandt E. (eds), pp. 80–136. Springer, Berlin, Germany.

Overmann, J. (2010). *Geomicrobiology: molecular and environmental perspective*, chapter Novel cultivation strategies for environmentally important microorganisms. Barton LL, Mandl M, Loy A (eds), pp. 69–89. Springer Science and Business Media B.V., Springer, Berlin, Germany.

Pedrós-Alió, C. (2006). Marine microbial diversity: can it be determined? *Trends Microbiol* **14**, 257–263.

Pinhassi, J., Sala, M. M., Havskum, H., Peters, F., Guadayol, O., Malits, A. and Marrase, C. (2004). Changes in bacterioplank-

ton composition under different phytoplankton regimens. *Appl Environ Microbiol* **70**, 6753–6766.

Poindexter, J. S. (1981). Oligothrophy - fast and famine existence. *Adv Microb Ecol* **5**, 63–89.

Port, A., Gurgel, K.-W., Staneva, J., Schulz-Stellenfleth, J. and Stanev, E. V. (2011). Tidal and wind-driven surface currents in the German Bight: HFR observations versus model simulations. *Ocean Dynamics* **61**, 1567–1585.

Postec, A., Urios, L., Lesongeur, F., Ollivier, B., Querellou, J. and Godfroy, A. (2005). Continuous enrichment culture and molecular monitoring to investigate the microbial diversity of thermophiles inhabiting deep-sea hydrothermal ecosystems. *Curr Microbiol* **50**, 138–144.

Postgate, J. R. and Hunter, J. R. (1964). Accelerated death of *Aerobacter aerogenes* starved in presence of growth-limiting substrates. *J Gen Microbiol* **34**, 459–473.

Rappé, M. S., Connon, S. A., Vergin, K. L. and Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630–633.

Ravenschlag, K., Sahm, K. and Amann, R. (2001). Quantitative molecular analysis of the microbial community in marine Arctic sediments (Svalbard). *Appl Environ Microbiol* **67**, 387–395.

Redfield, A. C. (1934). *James Johnstone memorial volume*, chapter On the proportions of organic derivations in sea water and their relation to the composition of plankton. Daniel R.J. (ed). University Press of Liverpool, Liverpool, UK.

Rees, M. (1996). Evolutionary ecology of seed dormancy and seed size. *Philos Trans R Soc Lond Ser B-Biol Sci* **351**, 1299–1308.

Reichenbach, H. (1981). Taxomony of the gliding bacteria. *Annu Rev Microbiol* **35**, 339–364.

Reichenbach, H. (1989). *Bergey's Manual of Systematic Bacteriology, Vol 3*, chapter Order I. *Cytophagales* Leadbetter 1974. Staley J.T., Bryant M.P., Pfennig N. and Holt J.G. (eds), pp. 2011–2013. Williams and Wilkins, Baltimore.

Reichenbach, H., Kohl, W., Bottgervetter, A. and Achenbach, H. (1980). Flexirubin-type pigments in *Flavobacterium*. *Arch Microbiol* **126**, 291–293.

Reise, K., Baptist, M., Burbridge, P., Dankers, N., Fischer, L., Flemming, B., P., O. A. and Smit, C. (2010). *Wadden Sea Ecosystem*, chapter The Wadden Sea – a universally outstanding tidal wetland., pp. 7–23. Common Wadden Sea Secretariat, Wilhelmshaven, Germany.

Riemann, L., Steward, G. F. and Azam, F. (2000). Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* **66**, 578–587.

Russell, H. L. (1891). Untersuchungen über im Golf von Neapel lebende Bakterien. *Zeitschrift für Hygiene und Infektionskrankheiten* **11**, 165–206.

Sait, M., Hugenholtz, P. and Janssen, P. H. (2002). Cultivation of globally distributed soil bacteria from phylogenetic lineages previously only detected in cultivation-independent surveys. *Environ Microbiol* **4**, 654–666.

Sakshaug, E., Andresen, K., Myklestad, S. and Olsen, Y. (1983). Nutrient status of phytoplankton communities in Norwegian waters (ma-

rine, brackish, and fresh) as revealed by their chemical composition. *J Plank Res* **5**, 175–196.

Sandy, M. and Butler, A. (2009). Microbial iron acquisition: marine and terrestrial siderophores. *Chem Rev* **109**, 4580–4595.

Schattenhofer, M., Fuchs, B. M., Amann, R., Zubkov, M. V., Tarran, G. A. and Pernthaler, J. (2009). Latitudinal distribution of prokaryotic picoplankton populations in the Atlantic Ocean. *Environ Microbiol* **11**, 2078–2093.

Schauer, R., Bienhold, C., Ramette, A. and Harder, J. (2010). Bacterial diversity and biogeography in deep-sea surface sediments of the South Atlantic Ocean. *ISME J* **4**, 159–170.

Schink, B. and Friedrich, M. (2000). Bacterial metabolism - phosphite oxidation by sulphate reduction. *Nature* **406**, 37.

Schut, F., Devries, E. J., Gottschal, J. C., Robertson, B. R., Harder, W., Prins, R. A. and Button, D. K. (1993). Isolation of typical marine bacteria by dilution culture: growth, maintenance, and characteristics of isolates under laboratory conditions. *Appl Environ Microbiol* **59**, 2150–2160.

Schut, F., Gottschal, J. C. and Prins, R. A. (1997). Isolation and characterisation of the marine ultramicrobacterium *Sphingomonas* sp. strain RB2256. *FEMS Microbiol Rev* **20**, 363–369.

Scripps Institution of Oceanography (1978). Probing the Oceans 1936 to 1976. *Tofua Press, San Diego, Calif.* **http://ark.cdlib.org/ark:/13030/kt109nc2cj/**.

Shipman, J. A., Berleman, J. E. and Salyers, A. A. (2000). Characterization of four outer membrane proteins involved in binding starch

to the cell surface of *Bacteroides thetaiotaomicron*. *J Bacteriol* **182**, 5365–5372.

Sieburth, J. M. (1967). Seasonal selection of estuarine bacteria by water temperature. *J Exp Mar Biol Ecol* **1**, 98–121.

Simon, M., Glöckner, F. O. and Amann, R. (1999). Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquat Microb Ecol* **18**, 275–284.

Staley, J. T. and Konopka, A. (1985). Measurements of in situ activities of nonphotosynthetic mikroorganisms in aquatic and terreestrial habitats. *Annu Rev Microbio* **39**, 321–346.

Stanier, R. (1947). Studies on nonfruiting myxobacteria I. *Cytophaga johnsonae*, n. sp., a chitin-decomposing myxobacterium. *J Bacteriol* **53**, 297–315.

Sterner, R. W., Andersen, T., Elser, J. J., Hessen, D. O., Hood, J. M., McCauley, E. and Urabe, J. (2008). Scale-dependent carbon : nitrogen : phosphorus seston stoichiometry in marine and freshwaters. *Limnol Oceanogr* **53**, 1169–1180.

Stevens, H., Simon, M. and Brinkhoff, T. (2009). Cultivable bacteria from bulk water, aggregates, and surface sediments of a tidal flat ecosystem. *Ocean Dynamics* **59**, 291–304.

Svedrup, H. U., Johanson, M. W. and Fleming, R. H. (1942). *The oceans-their physics, chemistry, and general biology.* Prentice-Hall, Englewood Cliffs , New Jersey.

Tan, T. L., Reinke, M. and Rüger, H. J. (1996). New dilution method in microtiter-plates for enumeration and enrichment of copiotrophic and oligotrophic bacteria. *Arch Hydrobiol* **137**, 511–521.

Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J. et al. (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336**, 608–611.

Thomas, F., Hehemann, J.-H., Rebuffet, E., Czjzek, M. and Michel, G. (2011). Environmental and gut *Bacteroidetes*: the food connection. *Front Microbiol* **2**, 93.

Thomas, J. B. and Goedheer, J. C. (1953). Relative efficiency of light absorbed by carotenoids in photosynthesis and phototaxis of *Rhodospirillum rubrum*. *Bioch Biophys Acta* **10**, 385–390.

Tyson, G. W., Lo, I., Baker, B. J., Allen, E. E., Hugenholtz, P. and Banfield, J. F. (2005). Genome-directed isolation of the key nitrogen fixer *Leptospirillum ferrodiazotrophum* sp. nov. from an acidophilic microbial community. *Appl Environ Microbiol* **71**, 6319–6324.

van Bodegom, P. (2007). Microbial maintenance: A critical review on its quantification. *Microb Ecol* **53**, 513–523.

Vasconcelos, M., Azenha, M. and Lage, O. M. (1996). Electrochemical evidence of surfactant activity of the HEPES pH buffer which may have implications on trace metal availability to cultures *in vitro*. *Anal Biochem* **241**, 248–253.

Vukusic, P. and Sambles, J. R. (2003). Photonic structures in biology. *Nature* **424**, 852–855.

Waksman, S. A. (1934). The role of bacteria in the cycle of life in the sea. *Scientific Monthly* **38**, 35–49.

Waksman, S. A., Carey, C. L., and Reuszer, H. W. (1933). Marine bacteria and their role in the cycle of life in the sea - I Decomposition of marine plant and animal residues by bacteria. *Biol Bull* **65**, 57–79.

Warren, R. A. J. (1996). Microbial hydrolysis of polysaccharides. *Annu Rev Microbiol* **50**, 183–212.

Weller, R., Glöckner, F. O. and Amann, R. (2000). 16S rRNA-targeted oligonucleotide probes for the *in situ* detection of members of the phylum *Cytophaga- Flavobacterim-Bacteroides*. *Syst Appl Microbiol* **23**, 107–114.

West, N. J., Obernosterer, I., Zemb, O. and Lebaron, P. (2008). Major differences of bacterial diversity and activity inside and outside of a natural iron-fertilized phytoplankton bloom in the Southern Ocean. *Environ Microbiol* **10**, 738–756.

Whiteley, A. S. and Bailey, M. J. (2000). Bacterial community structure and physiological state within an industrial phenol bioremediation system. *Appl Environ Microbiol* **66**, 2400–2407.

Whitesides, M. D. and Oliver, J. D. (1997). Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. *Appl Environ Microbiol* **63**, 1002–1005.

Widdel, F. and Bak, F. (1992). *The Prokaryotes Vol 2*, chapter Gram-negative mesophilic sulfate-reducing bacteria. Balows A., Trüper H.G., Dworkin M. and Harder W. (eds), pp. 3352–3378. Springer, Berlin, Germany.

Widdel, F., Kohring, G. W. and Mayer, F. (1983). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty-acids. III: Charac-

terization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. *Arch Microbiol* **134**, 286–294.

Widdel, F. and Pfennig, N. (1977). A new anaerobic, sporing, acetate-oxidizing, sulfate-reducing bacterium, *Desulfotomaculum* (emend.) *acetoxidans*. *Arch Microbiol* **112**, 119–122.

Williams, P., Winzer, K., Chan, W. C. and Camara, M. (2007). Look who's talking: communication and quorum sensing in the bacterial world. *Phil Trans R Soc B* **362**, 1119–1134.

Winkelmann, N. and Harder, J. (2009). An improved isolation method for attached-living *Planctomycetes* of the genus *Rhodopirellula*. *J Microbiol Methods* **77**, 276–284.

Woebken, D., Fuchs, B. M., Kuypers, M. M. M. and Amann, R. (2007). Potential interactions of particle-associated anammox bacteria with bacterial and archaeal partners in the Namibian upwelling system. *Appl Environ Microbiol* **73**, 4648–4657.

Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., Hooper, L. V. and Gordon, J. I. (2003). A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. *Science* **299**, 2074–2076.

Yanagita, T., Ichikawa, T., Tsuji, T., Kamata, Y., Ito, K. and Sasaki, M. (1978). Two trophic groups of bacteria, oligotrophs and eutrophs: their distributions in fresh and sea water areas in the central northern Japan. *J Gen Appl Microbiol* **24**, 59–88.

Yi, H. and Chun, J. (2012). Unification of the genera *Nonlabens*, *Persicivirga*, *Sandarakinotalea* and *Stenothermobacter* into a single emended

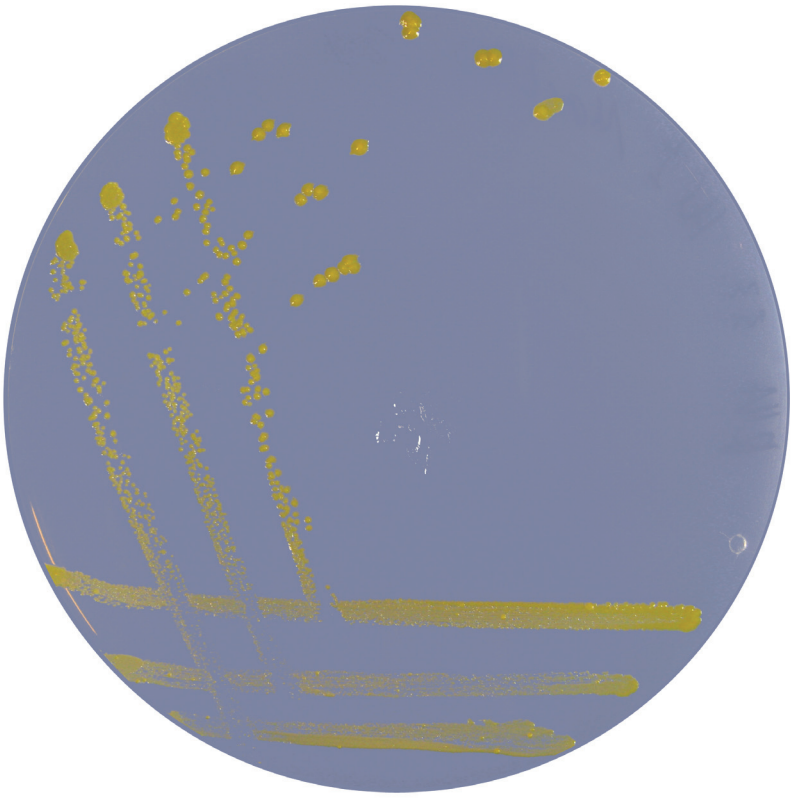
genus, *Nonlabens*, and description of *Nonlabens agnitus* sp. nov. *Syst Appl Microbiol* **35**, 150–155.

Zengler, K., Toledo, G., Rappé, M., Elkins, J., Mathur, E. J., Short, J. M. and Keller, M. (2002). Cultivating the uncultured. *Proc Natl Acad Sci USA* **99**, 15681–15686.

ZoBell, C. E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J Mar Res* **4**, 42–75.

ZoBell, C. E. (1946). *Marine microbiology. A monograph of hydrobacteriology.* Chronica Botanica Co., Waltham, Mass. U.S.A.

ZoBell, C. E. and Johnson, F. H. (1949). The influence of hydrostatic pressure on the growth and viability of terrestrial and marine bacteria. *J Bacteriol* **57**, 179–189.



Polaribacter strain cultivated on marine HaHa agar.

Chapter 2

Phylogenetic diversity of *Flavobacteria* isolated from the North Sea on solid media

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Contributions to the manuscript: R.L.H. and J.H. designed research and project outline, developed the new medium and the Flavobacteria-Cytophagia specific PCR assay, and conducted sampling on Sylt. R.L.H. organized and conducted sampling with the students at Harlesiel, Helgoland and Janssand, isolated and organized the students isolates, and performed phylogenetic analysis. R.L.H. and J.H. conceived, wrote and edited the manuscript.

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2.1 Abstract

Flavobacteria are abundant in the North Sea, an epeiric sea on the continental shelf of Europe. However, this abundance has so far not been reflected by strains in culture collections. We isolated *Flavobacteria* from pelagic and benthic samples, such as seawater, phytoplankton, sediment and its porewater, and from surfaces of animals and seaweeds on agar plates with a variety of carbon sources. Dilution cultivation with a new medium, incubation at low temperatures and with long incubation times, and a colony screening by a *Flavobacteria-Cytophagia* specific PCR detecting 16S rRNA gene sequences led to a collection of phylogenetically diverse strains. Besides two strains affiliating with *Flammevirgaceae* and seven strains affiliating with *Cyclobacteriaceae*, we isolated within the *Flavobacteriaceae* 20 strains presumably representing seven novel candidate genera and 355 strains affiliating with 26 of 80 validly described marine *Flavobacteriaceae* genera, based on a genus boundary of 95.0% 16S rRNA gene sequence identity. The majority of strains (276) affiliated with 37 known species in 16 genera (based on the boundary of 98.7% 16S rRNA gene sequence identity), whereas 79 strains likely represented 42 novel species in 22 established *Flavobacteriaceae* genera. Pigmentation, iridescence, gliding motility, agar lysis, and flexirubin as chemical marker supported the taxonomy on the species level. This study demonstrated the culturability of phylogenetically diverse *Flavobacteria* on solid medium originating from the North Sea.

2.2 Introduction

Flavobacteria are common in epipelagic oceanic and coastal waters as well as in benthic habitats, accounting for 10 to 30%, sometimes up to 70% of the bacterial populations (Eilers et al., 2000; Gómez-Pereira et al., 2010; Zinger et al., 2011). Together with *Alphaproteobacteria*, *Flavobacteria* were more abundant in the particle-associated fraction, whereas *Gammaproteobacteria* were dominantly free floating (Abell and Bowman, 2005). *Flavobacteria* are known to attach to phytoplankton (Gómez-Pereira et al., 2010) and to participate in the initial degradation of complex organic matter, thus playing an important part in the carbon cycle (Kirchman, 2002). In a decaying phytoplankton spring bloom in the North Sea, *Flavobacteria* populations dominated the initial degradation process (Teeling et al., 2012). The German Bight in the North Sea is a shallow coastal area with high tidal dynamics (Port et al., 2011) whose seafloor is a major sink of organic matter and nitrogen species (Alongi, 1998; Gao et al., 2012). In this coastal region, *Flavobacteria* were a dominating population in the microbial community in surface seawater, accounting for up to 55% of bacterioplankton cells (Eilers et al., 2000). In the benthos, *Flavobacteria* were the most abundant phylogenetic group, accounting for 15 to 25% of all cells (Llobet-Brossa et al., 1998). In 2006, the *Flavobacteriaceae* comprised 168 species in 53 genera (Bernardet, 2010). This family has risen to 393 species in 95 genera (www.bacterio.cict.fr, June 2012) (Euzéby, 1997). Marine strains represented 210 *Flavobacteriaceae* type strains in 80 genera (suppl. Tab. 2.S2).

In contrast to the population size, previous attempts to cultivate representatives of bacterial communities from the Wadden Sea obtained a low number of *Flavobacteriaceae* strains, irrespective whether they originated from seawater (Eilers et al., 2000) or intertidal sediment (Stevens et al., 2009). In both cases, polymeric carbohydrates (e.g. chitin, cellulose and

agar) did not support an increase in culturability. The authors concluded that (i) frequently isolated bacteria were of low abundance in nature (Eilers et al., 2000), and (ii) *Flavobacteria* did not grow well on solid agar (Stevens et al., 2009). Nevertheless, seven novel species of *Flavobacteriaceae* had been isolated from the North Sea and described in recent years. *Leeuwenhoekiella marinoflava* (Nedashkovskaya et al., 2005) was cultivated from the seawater of the coast of Aberdeen. *Maribacter forsetii* (Barbeyron et al., 2008) and '*Gramella forsetii*' (Bauer et al., 2006) were isolated from the seawater of Helgoland, an island in the German Bight. *Muricauda ruestringensis* was isolated from the intertidal sediment near the former village of Rüstringen (Bruns et al., 2001). *Tenacibaculum ovolyticum* was isolated from the epiflora of halibut eggs of Bergen, Norway (Hansen et al., 1992; Suzuki et al., 2001). *Tenacibaculum skagerrakense* was isolated from the seawater of Skagerrak, Denmark (Frette et al., 2004), and *Cellulophaga fucicola* from the brown algae *Fucus* of Hirsholm island, Denmark (Johansen et al., 1999).

The aim of our study was a collection of phylogenetically diverse *Flavobacteriaceae* from different locations and sample types of the German Bight of the North Sea. We explored improved techniques to isolate marine *Flavobacteria* using suitable medium components. A PCR with a *Flavobacteria-Cytophagia* specific primer for the 16S rRNA gene enabled a fast identification of *Flavobacteria* colonies.

2.3 Material and methods

Sampling

Samples were collected with Niskin bottles, 20 μm- or 80 μm-plankton nets, sterile syringes or tubes at Helgoland, Harlesiel, Janssand and the sites Königshafen, Hausstrand/List and Weststrand on Sylt (suppl. Tab.

2.S1 on page 86). Samples were stored at *in situ* temperature, transported to the laboratory within one to three hours and directly processed.

Medium preparation

Artificial seawater (ASW) and all media were prepared with sterile filtered (0.2 μm polycarbonate filter) ultra pure water (Aquintus system, membraPure, Germany) with a resistivity of 18.3 MΩ m. For dilutions and washing steps, ASW was prepared following the recipe of Widdel and Bak (1992) as described by Winkelmann and Harder (2009) (see suppl. on page 109). Basal salts: 26.37 g NaCl, 5.67 g $MgCl_2 \cdot 6\,H_2O$, 6.8 g $MgSO_4 \cdot 7\,H_2O$, 0.19 g $NaHCO_3$, 1.47 g $CaCl_2 \cdot 2\,H_2O$, 0.72 g KCl, 0.10 g KBr, 0.02 g H_3BO_3, 0.02 g $SrCl_2$ and 0.003 g NaF were dissolved in 1 L water. After autoclavation at 121 ℃ for 25 min and cooling, the ASW was slowly adjusted to pH 7.5 with autoclaved 1 M NaOH or 1 M HCl. Autoclaved water was used to replace the evaporated water. The ASW had a salinity of 34‰ S, comparable to the euhaline ($>$ 30‰ S) sampling sites. ZoBell (1941; 1946) suggested for the cultivation of most marine bacteria the marine medium 2216 with yeast extract (=2216E), which is nowadays sold as marine agar 2216. It was prepared following the manufacturer's instructions (Difco Laboratories, Detroit, USA). The evaporated water was replaced by autoclaved water. Other solid media with defined carbon sources required the preparation of twofold concentrated ASW and a purification of bacto agar (Difco Laboratories, Detroit, USA). Agar (18 g L^{-1}) was washed three times with 700 mL ultra pure water, to remove soluble substances that may inhibit bacterial growth (Widdel and Bak, 1992; Janssen et al., 2002). Solid HEPES (50 mM) and 500 mL twofold ASW were added to the agar suspension. After autoclavation, the medium was temperated at 55 ℃ and supplemented with 5 mL NH_4Cl (50 g L^{-1}, autoclaved), 10 mL KH_2PO_4 (50 g L^{-1}, autoclaved), 2 mL trace element solution (per L: $FeSO_4 \cdot 7\,H_2O$, 2.1 g; Na_2−EDTA, 5.2 g; H_3BO_3,

30 mg; $MnCl_2 \cdot 4\,H_2O$, 100 mg; $CoCl_2 \cdot 6\,H_2O$, 190 mg; $NiCl_2 \cdot 6\,H_2O$, 24 mg; $CuCl_2 \cdot 2\,H_2O$, 10 mg; $ZnSO_4 \cdot 7\,H_2O$, 144 mg; $Na_2MoO_4 \cdot 2\,H_2O$, 36 mg; pH adjusted to 6.0 with 5 M NaOH (Pfennig et al., 1981)), and 0.7 mL SeW solution (Widdel and Bak, 1992). Carbon sources for the SYL media were 2 g L^{-1} of yeast extract, peptone tryptone, casamino acids, glucose, cellobiose, N-acetylglucosamine, xylose, galactose, malate, arabinaose or rhamnose, for the HAR medium 0.3 g L^{-1} of casamino acids and 0.5 g L^{-1} of glucose, xylose and N-acetylglucosamine, and for the HaHa medium 0.5 g L^{-1} of yeast extract, peptone tryptone, casamino acids, glucose, and cellobiose. The SYL media received per liter 1 mL 7-vitamin solution (Winkelmann and Harder, 2009), 1 mL vitamin B_{12} solution (Widdel and Bak, 1992), 1 mL thiamine solution (Winkelmann and Harder, 2009), and 1 mL riboflavin solution (Winkelmann and Harder, 2009). The pH was slowly adjusted to 7.5 with autoclaved 1 M NaOH. Evaporated water was replaced with autoclaved water, before the plates were poured.

Isolation and cultivation

To enrich sediment-attached bacteria, 5 mL of the sediment from Harlesiel were sampled with a sterile cut-off syringe. The sediment was washed successively five times with 40 mL sterile artificial seawater in a 50 mL polypropylene tube; resulting in approximately 5.5×10^4 cells mL^{-1} sediment. Sediment was allowed to settle for 30 minutes and supernatant was decanted. The washed sediment was incubated in HAR liquid medium at 25 ℃ for 24 h. The sediment was mixed with the medium in an overhead rotator at 25 rpm (Reax 2, Heidolph, Schwabach, Germany). The next day, the sediment was washed five times with artificial seawater (40 mL) and afterward incubated for 48 h and 96 h in HAR liquid medium. The supernatant was decanted and collected in a fresh, sterile 50 mL polypropylene tube. Samples of the sediment or of the supernatant were incubated on solid

HAR medium. Kanamycin was reported to select for *Flavobacteria* (Flint, 1985). Surface intertidal sediments from Königshafen of Sylt, Janssand or Harlesiel were incubated on SYL agar, optionally supplemented with 50 μg mL^{-1} kanamycin (Flint, 1985) and incubated at 25 ℃ for 3–4 weeks. For inoculation, seawater aliquots were spread on solid agar plates using sterile glass beads and sediment was spread on agar plates with an inoculating loop. Algae were chopped and washed with sterile artificial seawater. Animal specimens were washed with seawater and sterile artificial seawater. The 96-pin replicator enabled a transfer of 1 μL per pin on 96 defined positions on a 150mm Petri dish with solid agar (Winkelmann and Harder, 2009). HAR and HaHa agar plates were incubated at 11 ℃ and SYL agar plates at room temperature (22 ℃) for at least two months. Single colonies were examined and three times transferred to new plates to obtain pure strains. Colonies were characterized by phenotypic characteristics as well as *Flavobacteria-Cytophagia* specific 16S rRNA gene amplification and sequence analysis. Strains were maintained as viable cultures on 2216 marine agar or on HaHa agar plates at +4 ℃ and also cryopreserved at –80 ℃, frozen within artificial seawater supplemented with 30% (v/v) glycerol.

16S rRNA gene analysis

Two protocols were applied to release DNA from cells. A tiny amount of a colony was dissolved from a sterile wooden toothpick in 20 μL PCR water. After three freeze/thaw cycles (–20 ℃ / +4 ℃), one microliter served as PCR template. Alternatively, the smallest separable part of the colony was squashed in 100 μL PCR water and lysed by three freeze/thaw cycles. The frozen sample finally received 100 μL PCR water and was thawed without mixing. Ten microliters of supernatant served as PCR template. The 16S rRNA gene was amplified with the general bacterial primers GM3F (5' -AGA GTT TGA TYM TGG CTC AG- 3') (positions 8-27 according

to *Escherichia coli* numbering) and 907R (5' -CCG TCA ATT CCT TTR AGT TT- 3') (Muyzer et al., 1995) as well as with the primers GM3F and CF1489R. The *Flavobacteria-Cytophagia* specific reverse primer CF1489R (5' -TAC CTT GTT ACG ACT TAG C- 3', positions 1489–1507) was designed and validated with the ARB software (Ludwig et al., 2004) on the dataset SILVA ref108_NR99 (Pruesse et al., 2007) and with SILVA TestPrime (Klindworth et al., 2012). PCR amplifications were performed in 25 μL with 96 ℃ for 4 min, 35 cycles of 96 ℃ for 1 min, 55 or 62 ℃ for 1 min –for primer pairs GM3F, 907R and GM3F, CF1489R, respectively–, 72 ℃ for 3 minutes and a final elongation at 72 ℃ for 10 min. The sequencing reaction applied the ABI Dye Terminator technology and an Applied Biosystems 3130xl DNAsequencer (Applied Biosystems). As an exception to standard conditions, GM3F-CF1489R amplicons were sequenced with an elongation temperature of 62 ℃, the optimal annealing temperature of CF1489R. The 16S rRNA gene sequences were analyzed with Applied Biosystems Sequencing Analysis 5.2 (Applied Biosystems, Foster City, USA) and assembled with Sequencer 4.6 (Gene codes, Ann Arbor, MI). The initial phylogenetic affiliation was assigned using the Ribosomal Database Project (Cole et al., 2009). After alignment of sequences in ARB, evolutionary distances were calculated by the method of Jukes and Cantor (1969) and a phylogenetic consensus tree was constructed with neighbour-joining (Saitou and Nei, 1987) using a 0% and 40% base frequency filter in ARB. The 16S rRNA gene sequences were deposited under Acc.No. JX854056 – JX854433.

Phenotypic characterization

The bathochromic shift test with 20% (w/v) KOH was performed to detect flexirubin type pigments (Fautz and Reichenbach, 1980) (suppl. Fig. 2.S5). Cell shapes were visualized with phase contrast microscopy. Shape and color of colonies on the agar plate were visualized with a binocular.

2.4 Results and Discussion

We isolated 375 strains affiliating with *Flavobacteriaceae* from all samples investigated: seawater of Helgoland, sediment of Harlesiel and Janssand, and seawater, sediment and its porewater, phytoplankton, seaweed and animal specimens of Sylt in the German Bight. The affiliation was based on the current nomenclature of *Flavobacteriaceae* (suppl. Tab. 2.S2). A novel species is defined by a 16S rRNA gene sequence identity between 95.0% and 98.7% (suppl. Tab. 2.S3), and a novel genus is defined as $<$ 95.0% 16S rRNA gene sequence identity with validly described *Flavobacteriaceae* (suppl. Tab. 2.S4) (Stackebrandt and Ebers, 2006; Yarza et al., 2010). The strains represented 7 novel genera, 42 novel species, and 37 validated species, including four species previously isolated from the North Sea; '*Gramella forsetii*' (Bauer et al., 2006), *Maribacter forsetii* (Barbeyron et al., 2008), *Muricauda ruestringensis* (Bruns et al., 2001) and *Cellulophaga fucicola* (Johansen et al., 1999). Detailed information on the strains is presented in Fig. 2.1 and suppl. Tab. 2.S3 to 2.S4 on pages 92–102.

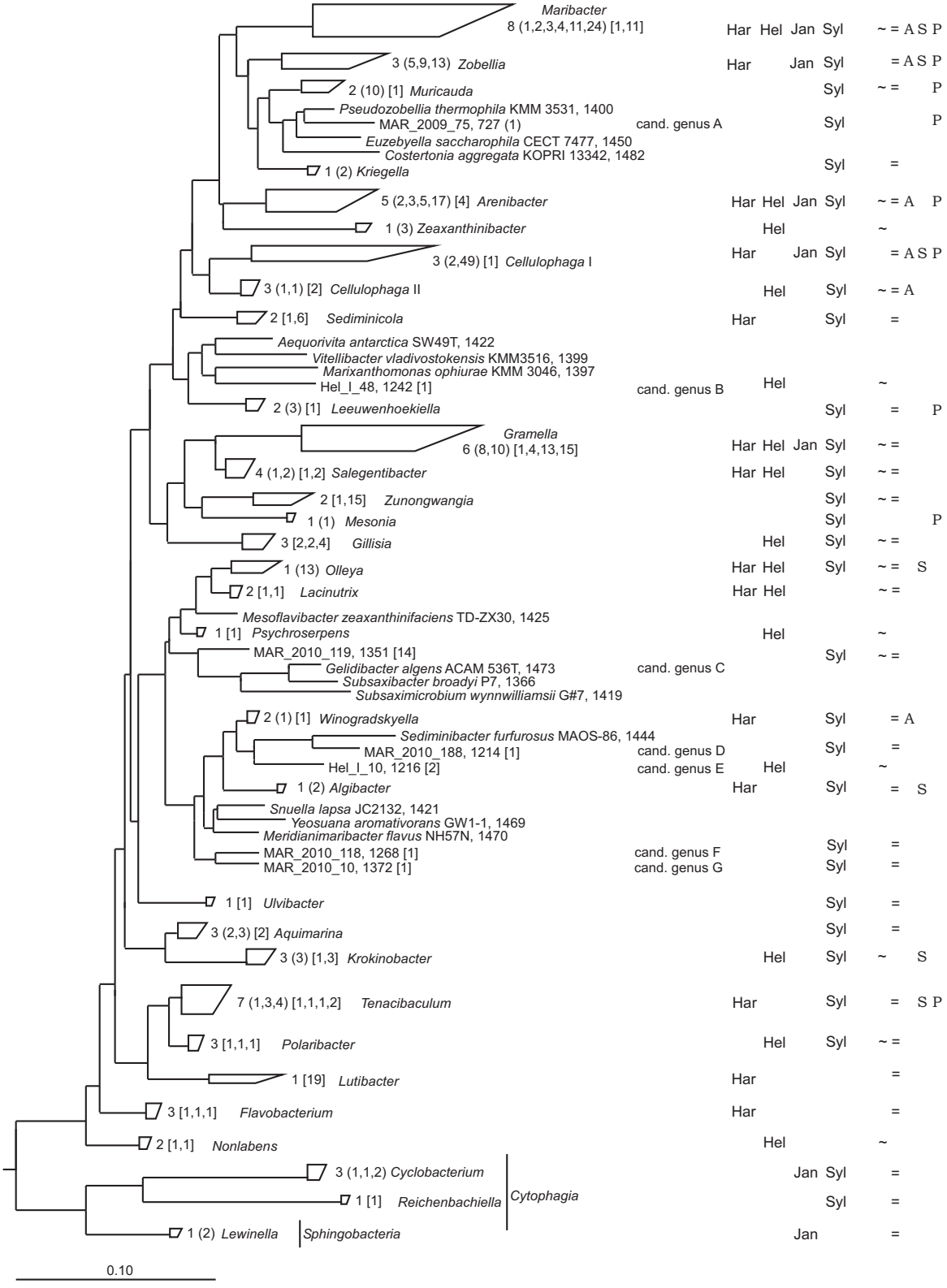
Selection criteria for isolation were initially the yellow colony color and a short rod-shaped to filamentous cell morphology. These selection criteria yielded a bias towards strains of the genera *Arenibacter*, *Cellulophaga* and *Maribacter* (suppl. Fig. 2.S2). A color-independent screen for the presence of *Flavobacteria* in colonies was developed with the *Flavobacteria-Cytophagia* specific reverse primer CF1489R. This primer

covered 86% of all *Bacteroidetes* sequences present the database SILVA ref108_NR99. The new primer CF1489R amplified in combination with the *Bacteria*-forward primer GM3F at 62 ℃ exclusively a nearly full length 16S rRNA gene of *Flavobacteria* or *Cytophagia*. The *Bacteria* specific primer GM3F and 907R revealed the presence of *Actinobacteria*, *Firmicutes*, *Alphaproteobacteria* and *Gammaproteobacteria* among the non-*Flavobacteria-Cytophagia* colonies. Thus, for aerobic marine samples the new primer is highly specific for *Flavobacteria*, *Sphingobacteria* and *Cytophagia*. Among the non-intensive yellow colonies detected as *Flavobacteria*, strains of *Zunongwangia*, 30 of 42 novel species, and 6 of 7 novel candidate genera were detected.

Variations in media and cultivation conditions

All strains were cultured as chemoheterotrophic bacteria on (i) ZoBell's 2216 marine agar or (ii) a defined artificial seawater medium supplemented with ammonium, phosphate, trace elements and as carbon and energy source with 2 g L^{-1} of complex carbon sources (yeast extract, peptone, casamino acids), defined carbohydrates (glucose, galactose, rhamnose, xylose, cellobiose, malate, or N-acetylglucosamine), or a mixture of both. HaHa medium was more suitable than marine agar 2216, partly because colonies of *Vibrio*, *Alteromonas* and *Pseudoalteromonas* were very large on 2216 and covered small adjacent colonies, but rarely formed colonies on HaHa medium. This may be due to the composition of the HaHa medium.

Figure 2.1 ***(facing page)*** Neighbour joining tree of *Flavobacteria*, based on nearly-complete 16S rRNA gene sequences (> 1100 bp) with parsimony addition of partial 16S rRNA gene sequences (< 1100 bp). Isolated strains originated from Harlesiel (Har), Helgoland (Hel), Janssand (Jan) or Sylt (Syl) and from seawater (∼), sediment (=), surfaces of animals (A), seaweed (S) or phytoplankton (P). The number preceding the bracket indicates the total number of species in the genus represented by isolated strains. The numbers in the round and square brackets indicate the number of strains affiliated to each species in the branch, separated by a comma. Square brackets indicate strains first identified by the *Flavobacteria-Cytophagia* specific PCR. Scalebar represents 10 nucleotide substitutions per 100 nucleotides.


Maribacter
8 (1,2,3,4,11,24) [1,11]
Har Hel Jan Syl ~ = A S P
3 (5,9,13) Zobellia
Har Jan Syl = A S P
2 (10) [1] Muricauda
Syl ~ = P
Pseudozobellia thermophila KMM 3531, 1400
MAR_2009_75, 727 (1)
cand. genus A
Syl P
Euzebyella saccharophila CECT 7477, 1450
Costertonia aggregata KOPRI 13342, 1482
1 (2) Kriegella
Syl =
5 (2,3,5,17) [4] Arenibacter
Har Hel Jan Syl ~ = A P
1 (3) Zeaxanthinibacter
Hel ~
3 (2,49) [1] Cellulophaga I
Har Jan Syl = A S P
3 (1,1) [2] Cellulophaga II
Hel Syl ~ = A
2 [1,6] Sediminicola
Har Syl =
Aequorivita antarctica SW49T, 1422
Vitellibacter vladivostokensis KMM3516, 1399
Marixanthomonas ophiurae KMM 3046, 1397
Hel_I_48, 1242 [1]
cand. genus B
Hel ~
2 (3) [1] Leeuwenhoekiella
Syl = P
Gramella
6 (8,10) [1,4,13,15]
Har Hel Jan Syl ~ =
4 (1,2) [1,2] Salegentibacter
Har Hel Syl ~ =
2 [1,15] Zunongwangia
Syl ~ =
1 (1) Mesonia
Syl P
3 [2,2,4] Gillisia
Hel Syl ~ =
1 (13) Olleya
Har Hel Syl ~ = S
2 [1,1] Lacinutrix
Har Hel ~ =
Mesoflavibacter zeaxanthinifaciens TD-ZX30, 1425
1 [1] Psychroserpens
Hel ~
MAR_2010_119, 1351 [14]
Syl ~ =
Gelidibacter algens ACAM 536T, 1473
Subsaxibacter broadyi P7, 1366
Subsaxicimicrobium wynnwilliamsii G#7, 1419
cand. genus C
2 (1) [1] Winogradskyella
Har Syl = A
Sediminibacter furfurosus MAOS-86, 1444
MAR_2010_188, 1214 [1]
cand. genus D
Syl =
Hel_I_10, 1216 [2]
cand. genus E
Hel ~
1 (2) Algibacter
Har Syl = S
Snuella lapsa JC2132, 1421
Yeosuana aromativorans GW1-1, 1469
Meridianimaribacter flavus NH57N, 1470
MAR_2010_118, 1268 [1]
cand. genus F
Syl =
MAR_2010_10, 1372 [1]
cand. genus G
Syl =
1 [1] Ulvibacter
Syl =
3 (2,3) [2] Aquimarina
Syl =
3 (3) [1,3] Krokinobacter
Hel Syl ~ S
7 (1,3,4) [1,1,1,2] Tenacibaculum
Har Syl = S P
3 [1,1,1] Polaribacter
Hel Syl ~ =
1 [19] Lutibacter
Har =
3 [1,1,1] Flavobacterium
Har =
2 [1,1] Nonlabens
Hel ~
3 (1,1,2) Cyclobacterium
Jan Syl =
1 [1] Reichenbachiella
Cytophagia
Syl =
1 (2) Lewinella
Sphingobacteria
Jan =
0.10

For a spring sample with a temperature of 6.4 ℃, we performed the isolation and cultivation at 11 ℃. This experiment yielded strains representing 11 of 42 novel *Flavobacteriaceae* species and 3 of 7 novel candidate genera in our study, but only one of 37 known species. Even though the environment reaches mesophilic temperatures (20 ℃) during the year, the observed shift towards novel species observed in cultivations at low temperature, near the *in situ* temperature, highlights the temperature as an important variable in isolation experiments.

Inoculation on plates was performed with traditional spreading techniques. Alternatively, we spotted one microliter on the plate using a 96-pin replicator (Winkelmann and Harder, 2009). Twofold dilution series yielded plates with high numbers of single colonies per inoculation spot. To determine the time for colony formation, a spring pelagic water sample from Helgoland with an *in situ* temperature of 6.4 ℃ was diluted and 1152 spots of 0.1 μL original seawater sample were observed for growth at 12 ℃ for 300 days (Tab. 2.1). The CFU increased during the incubation time, comparable to a growth curve. After a lag phase of 10 days, the number of colony forming units (CFU) exponentially increased till day 23 to 153 CFU and accumulated to 208 CFU at day 110. Besides *Actinobacteria* (e.g. *Rhodococcus*, *Nocardioides*), *Alphaproteobacteria* (*Erythrobacter*, *Sulfitobacter* and *Brevundimonas*) and *Gammaproteobacteria* (e.g. *Marinobacter*, *Pseudoalteromonas*), we obtained 88 yellow-orange to brownish pigmented colonies and among them 43 *Flavobacteriaceae*, correspondingly 273 CFU per mL seawater. The first *Flavobacteriaceae* colonies were strains of *Krokinobacter*, *Croceibacter*, *Maribacter* and *Salegentibacter*. Also strains affiliating with *Gillisia*, *Stenothermobacter*, *Arenibacter* and *Marixanthimonas* required less than 20 days to form visible colonies. In contrast, strains of *Cellulophaga*, *Flavobacterium* and *Nonlabens* required at least 20 days for colony formation (Fig. 2.2). Several strains of novel species required long

incubation times of several weeks, whereas over 80 percent of viable cells needed only three weeks to grow to visible colonies. The incubation time seems to be an important factor for the cultivation of novel species.

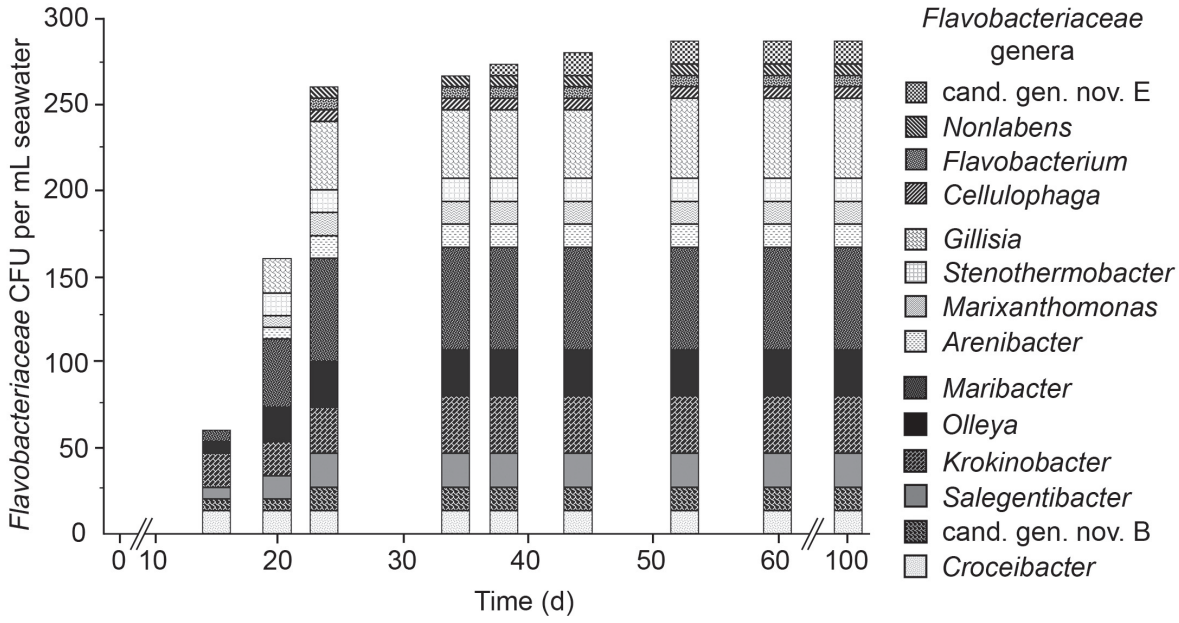


Figure 2.2 Colony formation (CFU per mL seawater) of strains affiliating with *Flavobacteriaceae* genera. The seawater of Helgoland Roads of 20 April 2010 was incubated on HaHa medium at 11 ℃ for 300 days.

Ken P. Flint (1985) suggested kanamycin as effective agent to enhance the culturability of 'Flavobacterium' species by growth inhibition of other bacteria. Plates with 2 g L^{-1} casamino acids were supplemented with 50 μg mL^{-1} kanamycin and inoculated with phytoplankton or sediment samples. In comparison with control plates, the number of white colonies was reduced by 50% on average, whereas the number of yellow colonies remained nearly constant (suppl. Fig. 2.S3). The *Flavobacteria-Cytophagia* specific PCR was positive for 90% of the yellow colonies. This experiment confirmed the observations of Flint (1985) and the resistance of many *Flavobacteria* to the aminoglycoside antibiotic kanamycin. Strains obtained from kanamycin-containing media affiliated with *Arenibacter* (1 strain), *Cellulophaga* (7), *Gramella* (6), *Kriegella* (1), *Lutibacter* (15), *Maribacter* (1), *Mesonia* (1),

Table 2.1 Colony formation (CFU per milliliter seawater) of a Helgoland spring seawater sample (20 April 2010) on HaHa medium incubated at 11 °C.

Time (days)	15	20	23	34	38	44	52	60	100	110	300
Total CFU	227	533	1020	1100	1160	1213	1267	1320	1347	1387	1387
Pigmented CFU	167	333	507	547	567	573	587	587	587	587	587
Flavobacteria CFU	60	160	260	267	267	267	273	273	273	273	273

Muricauda (2), *Saligentibacter* (1), *Sediminicola* (1), *Tenacibaculum* (2), *Winogradskyella* (1), and *Zobellia* (9). Thus, a selective isolation of certain genera was not observed by the application of kanamycin.

Biogeography and culturability

The genera *Cellulophaga*, *Maribacter*, *Gramella*, *Arenibacter*, *Lutibacter*, *Zunongwangia*, *Olleya*, *Zobellia*, and *Muricauda* were isolated frequently, with more than ten strains per species. *Zunongwangia profunda* (15 strains) and *Gramella echinicola* (13 strains) were exclusively isolated from one sampling site, in this case, from the porewater 1.5 m below the surface of West Beach, Sylt. Strains affiliating with *Lutibacter litoralis* (19 strains) or *Maribacter stanieri* (11 strains) were only isolated from the sediment of Harlesiel or the seawater of Helgoland, respectively. All other strains affiliating with one species –as defined by the 16S rRNA gene sequence identity– were isolated from two or more sampling sites and types. This reflects either the low resolution of the 98.7% 16S rRNA gene sequence identity used as boundary or the lack of a biogeography of many *Flavobacteria* in the German Bight.

In our study, the culturability of *Flavobacteria* from the North Sea on solid media is still approximately one colony forming unit among thousand flavobacterial cells. We cultivated strains of 26 known *Flavobacteriaceae* genera. Previous studies had isolated strains of seven genera, *Flavobacterium*, *Gillisia*, *Krokinobacter*, *Nonlabens*, *Polaribacter*, *Tenacibaculum* and *Winogradskyella* (Eilers et al., 2000; Teske et al., 2000; O'Sullivan et al., 2006; Wichels et al., 2006; Stevens et al., 2009; Riedel et al., 2010),

and corresponding 16S rRNA gene sequences were detected in cultivation independent studies (Zubkov et al., 2001; Alonso et al., 2007; Sapp et al., 2007; Rink et al., 2008; Teeling et al., 2012). Furthermore, strains of the genera *Algibacter*, *Aquimarina*, *Arenibacter*, *Cellulophaga*, *Gramella*, *Leeuwenhoekiella*, *Maribacter* and *Zobellia* had been cultivated, but were not present in cultivation independent studies (Eilers et al., 2000; Grossart et al., 2004; Stevens et al., 2005; Bauer et al., 2006; O'Sullivan et al., 2006; Wichels et al., 2006; Stevens et al., 2009; Salaün et al., 2010). The 16S rRNA gene sequences of *Marixanthimonas* and *Psychroserpens* were found in cultivation independent studies only (Musat et al., 2006; Brandt et al., 2010). These observations showed that we have broadened the diversity of culturable *Flavobacteria* from the North Sea (Eilers et al., 2000; Stevens et al., 2009) and, in contrast to previous reports, a wide range of *Flavobacteria* grew well on solid agar media, but many important taxa still await cultivation.

Physiological and chemotaxonomic observations

The known types of gliding motility were observed (Fig. 2.3): (i) spreading as thin film or as waves (*Cellulophaga*, *Tenacibaculum*), (ii) an outwards push even around the colony (*Leeuwenhoekiella*, *Polaribacter*, *Zobellia*) or (iii) an flame-like pattern (*Aquimarina*, *Krokinobacter*, *Pseudozobellia*, *Zobellia*), and (iv) a rhizoid spreading along the streaking (*Algibacter*, *Gramella*, *Maribacter*, *Zeaxanthinibacter*). For *Krokinobacter* spp., movement by gliding was not determined, but putative gliding-related proteins were encoded in the genome of *Krokinobacter* sp. 4H-3-7-5 (Klippel et al., 2011). Strain SRO_11 affiliating with *Krokinobacter eikastus* glided on marine agar 2216.

Iridescence (Vukusic and Sambles, 2003; Doucet and Meadows, 2009; Meadows et al., 2009) was briefly described among *Flavobacteria*

(Bernardet, 2010; Kientz et al., 2012), but intensively for *Cellulophaga* (Kientz et al., 2012). We observed iridescence in strains affiliated with the genera *Cellulophaga*, *Algibacter* and *Maribacter*. Flexirubin-type pigments (Fautz and Reichenbach, 1979) were detected in coherence with the species description in strains affiliating with *Aquimarina*, *Kriegella*, and *Zobellia*. Unexpectedly, strains MGE_SAT_544_1 and MAR_2010_101 among 31 strains of *Arenibacter* showed the bathochromic shift after KOH treatment. In contrast, the *Ulvibacter* strain MAR_2010_11 had no flexirubin-type pigment. Besides these exceptions, pigmentation, iridescence, gliding motility, agar lysis, and flexirubin as chemical marker supported the taxonomy on the species level.

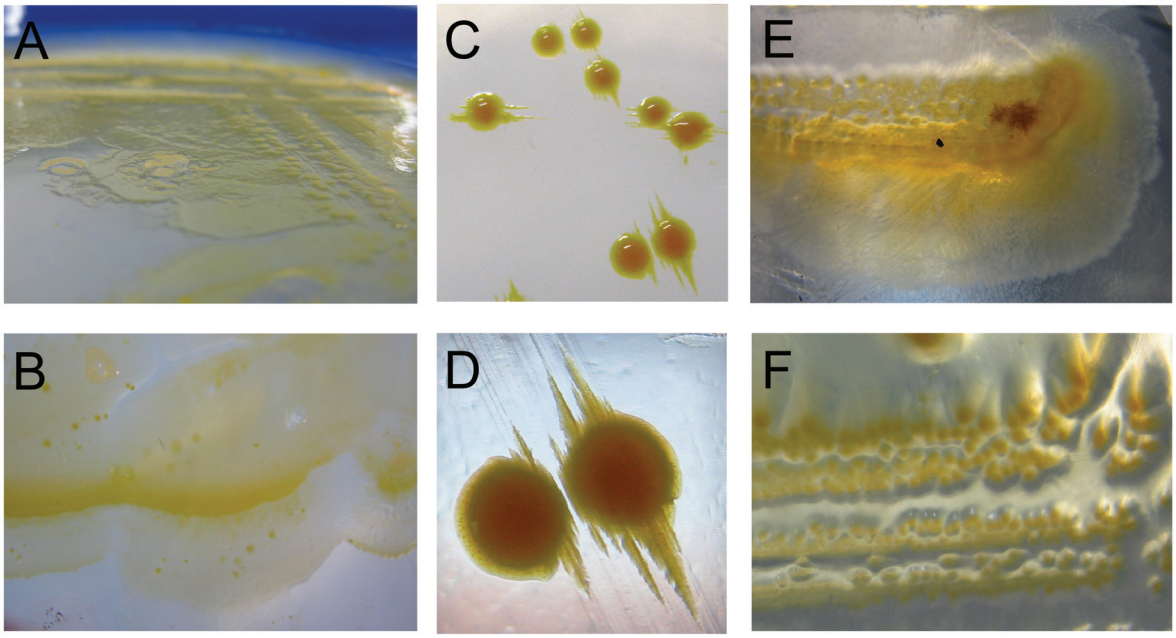


Figure 2.3 Gliding modes among *Flavobacteriaceae* strains isolated from North Sea samples. Cultures of *Cellulophaga* produced a thin film on the agar surface (A, *Cellulophaga* sp. RHA_28) or biofilm waves (B, *Cellulophaga* sp. MAR_2009_44). Cultures of *Gramella* glided a little from the colony away along the streak (C, D, *Gramella* sp. MAR_2010_21). Cultures of *Zobellia* pushed outwards from around the colony (E, *Zobellia* sp. MAR_2009_186) or in a flame-like pattern (F, *Zobellia* sp. RHA_40) to new areas of the medium.

Zobellia, candidate genus A, *Muricauda* and *Kriegella*

Twenty-eight strains were isolated affiliating with three *Zobellia* spp., *Z. uliginosa*, *Z. amurskyensis* and *Z. russellii*. All strains were positive for flexirubin-type pigments. Three strains of the species *Z. russellii* and one strain of *Z. uliginosa* lysed agar. Seven strains of all three species showed gliding motility in a flame-like pattern or even around the colony (Fig. 2.3). Ten strains affiliated with *Muricauda ruestringensis*. These were flexirubin-type pigment negative and did not glide or lyse agar, except for strain MAR_2009_54. Both strains of *Kriegella* were flexirubin-type pigment positive, but showed no gliding. The strain MAR_2009_75 had a 16S rRNA gene sequence identity of 94.7% to the closest relative *Pseudozobellia thermophile*, thus representing the candidate genus A. Iridescence, agar lysis and flexirubin-type pigments were not observed. This strain was isolated from phytoplankton of Sylt and showed a flame-like gliding pattern on agar (Fig. 2.3).

Sediminicola, candidate genus B, and *Leeuwenhoekiella*

Seven strains isolated from sediment were assigned to *Sediminicola luteus* and a novel *Sediminicola* sp.. No flexirubin-type pigments, gliding, agar lysis and iridescence were observed. Four strains of two novel species of *Leeuwenhoekiella* showed no flexirubin-type pigments, gliding, agar lysis and iridescence, but strain SRO_13 exhibited gliding motility. One strain isolated from the seawater of Helgoland had only a 90.4% 16S rRNA gene sequence identity with its closest relative being *Marixanthomonas ophiurae* and thus represents candidate genus B. Agar lysis, flexirubin-type pigments or iridescence were not observed, but gliding cells were observed around colonies.

Maribacter

Fifty-seven strains were assigned to eight species within *Maribacter*, including *M. stanieri*, *M. dokdonensis*, *M. sedimenticola*, and *M. forsetii* (Barbeyron et al., 2008) –a species previously isolated from Helgoland– and four novel species. Two strains of *M. dokdonensis* showed a glitter-like iridescence on the surface of the colony and a gliding following the streaking in a rhizoid spreading. Non of the strains produced flexirubin-like pigments.

Gramella

Thirty-two strains were isolated from sediment affiliating with *G. echinicola*, *G. gaetbulicola*, *G. marina* and a novel species of *Gramella*. Nineteen strains were isolated from seawater and sediment affiliating with *G. portivictoriae* and '*G. forsetii*' (Bauer et al., 2006). Gliding was observed for three strains which moved from the colony along the streak (Fig. 2.3). Iridescence, agar lysis and flexirubin-type pigments were not observed.

Arenibacter and *Zeaxanthinbacter*

Thirty-one strains were assigned to *Arenibacter*, including *A. troitsensis*, *A. palladensis*, *A. echinorum* and two novel *Arenibacter* spp. Gliding, agar lysis and iridescence were not observed. Among thirty-one strains, strains MAR_2010_101 and MGE_SAT_544_1 were flexirubin-type pigments positive. Three strains affiliating with *Zeaxanthinibacter enoshimensis* did not show iridescence, flexirubin-type pigments and agar lysis, but glided along the streaking (Fig. 2.3).

Cellulophaga

Fifty-six strains of *Cellulophaga* were isolated from sediment, seawater, biofilm, seaweed and animals of all sampling sites, and formed a monophyletic branch of two distinct subgroups in the 16S rRNA gene tree as

described by Bernardet (2010). In the first subgroup, 52 strains were affiliated with *C. lytica* or represented two novel *Cellulophaga* spp.. In the second subgroup, four strains were affiliated with *C. baltica*, *C. pacifica* or represented another novel *Cellulophaga* sp.. Gliding movement was observed for *C. lytica* and *Cellulophaga* sp. nov. I strains, with a faster spreading on the agar plate than colony formation, resulting in a thin film on the agar plate (Fig. 2.3). These cultures were isolated by serial dilution in artificial seawater followed by a homogenous distribution on an agar plate. Twelve strains of *C. lytica* and strain RHA_19 showed a glitter-like iridescence.

Salegentibacter, *Zunongwangia*, *Mesonia*, and *Gillisia*

Seven strains were assigned to *Salegentibacter mishustinae*, *S. salarius*, and two novel *Salegentibacter* spp.. Sixteen strains with a cell size of less than 1.5 μm and a faint yellow colony color were assigned to *Zunongwangia profunda* and one novel *Zunongwangia* sp.. *Mesonia algae* was represented by one strain. In *Gillisia*, 8 strains were affiliated with *G. mitskevichiae*, *G. myxillae* or depicted a novel species of *Gillisia*. The strains of *Salegentibacter*, *Zunongwangia*, *Mesonia*, and *Gillisia* were negative for flexirubin-type pigments, iridescence, agar lysis and gliding.

Flavobacterium and *Nonlabens*

Three strains were isolated from sediment affiliating with three species of *Flavobacterium*, *F. gelidilacus*, and two *Flavobacterium* sp. nov.. Two strains isolated from seawater of Helgoland represented novel species of the genus *Nonlabens*. Gliding, iridescence, agar lysis and flexirubin-type pigments were not observed.

Olleya, *Lacinutrix*, *Psychroserpens* and candidate genus C

Thirteen strains represented a novel species of *Olleya*, two strains were assigned to *Lacinutrix copepodicola* and *Lacinutrix* sp. nov., and one strain to a novel *Psychroserpens* sp.. Thirteen strains isolated from the porewater 1.5 m below the sand surface at the driftline at Sylt West Beach represented the candidate genus C, with a 16S rRNA gene sequence identity of 94.0% with *Gelidibacter algens*. The strains of *Olleya*, *Lacinutrix*, *Psychroserpens*, and the candidate genus C were negative for flexirubin-type pigments, iridescence, agar lysis and gliding.

Winogradskyella, *Algibacter* and candidate genera D, E, F, and G

Four strains represented two novel *Winogradskyella* spp. and two novel *Algibacter* spp.. Three strains were assigned to the candidate genera D and E, with a 16S rRNA gene sequence identity of 94.0% and 91.4% to the next relative *Sediminibacter furfurosus*, respectively. Two strains represented the candidate genera F and G, with the next relative *Meridianimaribacter flavus* with a 16S rRNA gene sequence identity of 94.7% and 95.0%, respectively. Gliding, agar lysis, iridescence and flexirubin-type pigments were not observed for strains of *Winogradskyella* and the candidate genera D, E, F, and G. For *Algibacter* strains, iridescence and gliding along the streaking (Fig. 2.3) was observed.

Ulvibacter, *Aquimarina* and *Krokinobacter*

One strain represented a novel species of *Ulvibacter*. This strain did not produce flexirubin-type pigments, in contrast to the current description of the genus *Ulvibacter*. Seven strains with more than 10 μm long cells grouped into three species of *Aquimarina*, *A. macrocephali* and two novel *Aquimarina* spp.. The flexirubin test was positive for strains of two novel species of *Aquimarina*, but not for strains of *A. macrocephali*. Gliding

motility was observed for strains of *A. macrocephali* and *A.* sp. nov. II, isolated from sediment, but not for strains of *A.* sp. nov. II, isolated from seawater. Seven strains affiliated with *Krokinobacter*, including *K. eikastus* and two novel *Krokinobacter* spp.. The strain SRO_199 performed a flame-like gliding and for the strain SRO_18 agar lysis was observed.

Tenacibaculum, *Polaribacter* and *Lutibacter*

Thirteen strains were isolated from seawater, sediment, phytoplankton and algae affiliating with *Tenacibaculum gallaicum*, *T. litoreum*, or presented 5 novel species of *Tenacibaculum*. Strains of *T. gallaicum* and *T.* sp. nov. II performed gliding which was faster than colony formation, resulting in a thin film on the agar plate (Fig. 2.3). Three novel species of *Polaribacter* were isolated. The strain Hel1_85 performed gliding, even around the colony. Nineteen strains from the sediment of Harlesiel were affiliated with *Lutibacter litoralis*. In contrast to the cell size of less than 1.5 μm of *Polaribacter* and *Lutibacter* strains, the *Tenacibaculum* strains formed filaments of more than 100 μm length.

Cytophagia and *Sphingobacteria*

An orange to brown colony color and rod-shaped cells characterized non-motile strains affiliating to *Reichenbachiella* (family *Flammeovirgaceae*), *Lewinella* (family *Saprospiraceae*) and *Cyclobacterium* (family *Cyclobacteriaceae*).

Conclusion and future perspectives

In this study we demonstrated the cultivation of marine *Flavobacteriaceae* on agar plates from diverse habitats. A broad phylogenetic diversity was obtained by different cultivation approaches for pelagic and benthic *Flavobacteria*, a *Flavobacteria-Cytophagia* specific PCR, and a suitable medium.

This collection of *Flavobacteriaceae* from the German Bight of the North Sea provides model organisms of marine aerobic heterotrophic bacteria and will give access to a variety of carbohydrate active enzymes (Cantarel et al., 2009; Teeling et al., 2012).

2.5 Acknowledgments

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2.6 Supporting Information

Figures, Tables and Methods

Phylogenetic diversity of *Flavobacteria* isolated from the North Sea on solid media

Richard L. Hahnke and Jens Harder

Table 2.S1 Sampling Sites.

	Sampling						Transport			Comment
Place	Latitude	Longitude	Time	Depth	Tide	T (°C)	Type	T (°C)	Duration (h)	
Seawater samples										
Helgoland Roads	54° 11' 03" N	7° 54' 00" E	20.04.2010	0–1 m	high	6	1L Schott bottle	10	1	pH 7.8, untreated
Helgoland Roads	54° 11' 03" N	7° 54' 00" E	10.06.2010	0–1 m	high	13	1L Schott bottle	10	1	untreated
Sylt, List	55° 01' 32" N	8° 27' 23" E	20.10.2010	0–1 m	high	14	50 mL PP tubes	14	1	untreated
Phytoplankton										
Sylt, List	55° 01' 32" N	08° 27' 23" E	16.10.2009	0–1 m	high	10	50 mL PP tubes	10	1	80 μm or 20 μm plankton net
Porewater										
Sylt – West Beach	55° 02' 01" N	08° 23' 07" E	21.10.2010	1.5 m	high	14	50 mL PP tubes	14	1	porewater of approaching tide
Sediment										
Janssand – middle flat[a]	53° 44' 12" N	07° 41' 55" E	25.10.2009	0–1 cm	low	9	50 mL PP tubes	10	3	black below 1.5 – 2 cm depth
Janssand – upper flat[a]	53° 44' 6" N	07° 41' 57" E	25.10.2009	0–1 cm	low	9	50 mL PP tubes	10	3	black below 1.5 – 2 cm depth
Harlesiel	53° 42' 48" N	07° 48' 23" E	11.02.2010	0–1 cm	low	-1	50 mL PP tubes	4	3	black below 8 – 10 cm depth
Sylt – West Beach	55° 02' 01" N	08° 23' 07" E	20.10.2008	0–1 cm	low	15	50 mL PP tubes	15	1	black below 8 – 10 cm depth
Sylt – Hausstrand	55° 00' 54" N	08° 26' 13" E	16.10.2009	0–1 cm	low	13	50 mL PP tubes	13	1	black below 8 cm depth
Sylt – Königshafen	55° 02' 15" N	08° 24' 43" E	16.10.2009	0–1 cm	low	13	50 mL PP tubes	13	1	black below 3 cm depth
Seaweed and animal specimen										
Sylt – Harbor	55° 01' 58" N	08° 26' 26" E	16.10.2009		low	14	bucket	14	1	*Fucus*
Sylt – national park	55° 01' 22" N	08° 26' 25" E	16.10.2009		low	14	bucket	14	1	*Polysiphonia*, *Ulva*, Crab, Starfish, Jellyfish, *Lanice*

[a]for more information about Janssand see (Gao et al., 2012)

Table 2.S2 *Flavobacteriaceae* genera with type strains, their environmental type (mar, marine; ter, terrestrial; fre, freshwater; cli, clinical), relation to other organisms (FL, free living; S, saprophytic; P parasitic), pigmentation (F+, flexirubin type pigments; F- no flexirubin type pigments; C carotinoids; Pig-, no pigmentation; nd, not determined), gliding movement (gl, gliding; d, gliding varies among type strains), number of type strains in each genus (2006, listed in (Bernardet, 2010); 2012, listed in (http://www.bacterio.cict.fr, 06.2012) (Euzéby, 1997); number of type strains with marine life style (Sed, sediment; SW, seawater; S, saprophytic; P, parasitic), and reclassification as other genus (⟶).

Genus	Type strain	Envir.	Rel.	Isolation source	Pig.	Gl.	2006	2012	Sed	SW	S	P
Actibacter	*A. sediminis*	mar	FL	tidal flat sediment	nd		0	1	1	0	0	0
Aequorivita	*A. antarctica*	mar, ter	FL, S	sea water, sea-ice algal, quartz stone subliths	C		4	5	0	3	1	0
Aestuariibaculum	*A. suncheonense*	mar	FL, S	tidal flat sediment	C		0	1	1	0	0	0
Aestuariicola	*A. saemankumensis*	mar	FL	tidal flat sediment	nd		0	1	1	0	0	0
Algibacter	*A. lectus*	mar	S	green algae *Acrosiphonia sonderi* (Kütz) Kornm and *Ulva fenestrata* Ruprecht	F-	gl	1	2	0	0	2	0
Aquimarina	*A. muelleri*	mar	FL, S	sea water, alga, marine sponge, sea urchin, tidal flat sediment	F+	gl	1	9	2	5	5	0
Arenibacter	*A. latericius*	mar	FL, S	sandy sediment samples, green alga, sea urchin	C	d	4	7	3.5	0	3.5	0
Aureicoccus	*A. marinus*	mar	FL	seawater	C		1	1	0	1	0	0
Aureitalea	*A. marina*	mar	FL	seawater	C		1	1	0	1	0	0
Aureivirga	*A. marina*	mar	FL, S	sponge Axinella verrucosa	C		0	1	0	0	1	0
Bergeyella	*B. zoohelcum*	cli	P, S	animal wounds	P-		1	1				
Bizionia	*B. paragorgiae*	mar	FL, S	soft coral, sea urchin, seawater, sea-ice brine, algae-feeding amphipod	C		5	8	0	4	4	0
Capnocytophaga	*C. ochracea*	cli	P, S	dog bite, human dental plaque and sputum	F+	gl	7	8				
Cellulophaga	*C. lytica*	mar	FL, S	beach mud, seawater, algal	C	gl	5	7	2	2	3	0
Chryseobacterium	*C. gleum*	cli, mar, ter, fre	FL, S, P	human and animal surface clinical samples, marine, soil, water reservoir, water-cooling system, wastewater, freshwater lake, raw cow's milk, midgut of the mosquito, rhizosphere plants, marine mud, seawater, permafrost, deep ice core, beer-bottling plants	F+		19	63	1	1	5	0
Cloacibacterium	*C. normanense*	fre	FL	municipal wastewater, freshwater lake sediment	C		0	2				
Coenonia	*C. anatina*	cli	P	ducks and geese	P-		1	1				
Corallibacter	*C. vietnamensis*	mar	S	coral *Palythoa* sp.	nd	gl	0	1	0	0	1	0
Costertonia	*C. aggregata*	mar	FL	mature biofilm	C		0	1	1	0	0	0
Croceibacter	*C. atlanticus*	mar	FL, S	sea water	C		1	1	0	0.5	0.5	0
Croceitalea	*C. eckloniae*	mar	S	alga, rhizosphere of the marine alga	C		0	2	0	0	2	0
Dokdonia	*D. donghaensis*	mar	FL	sea water	F-		1	1	0	1	0	0

Continued on next page

Table 2.S2 (continued)

Genus	Type strain	Envir.	Rel.	Isolation source	Pig.	Gl.	2006	2012	Sed	SW	S	P
Donghaeana	*D. dokdonensis*	⟶ *Persicivirga dokdonensis*					1	0				
Elizabethkingia	*E. meningoseptica*	cli, ter	FL, S, P	meningitis and septicaemia, midgut of the mosquito, Mir space station	P-		2	3				
Empedobacter	*E. brevis*	cli	P, S	various sources including canal water, clinical specimens, food, fish and marine animals, dogs, and pro	F+		1	1				
Epilithonimonas	*E. tenax*	fre	FL, S	epilithon-covered stones, raw cow's milk	F+		1	2				
Eudoraea	*E. adriatica*	mar	FL	coastal waters			0	1	0	1	0	0
Euzebyella	*E. saccharophila*	mar	FL	seawater	C		0	1	0	1	0	0
Flagellimonas	*F. eckloniae*	mar	S	rhizosphere of the marine alga *Ecklonia kurome*	C		0	1	0	0	1	0
Flaviramulus	*F. basaltis*	mar	FL	seafloor basalt, depth of 1300 m	C		0	1	1	0	0	0
Flavivirga	*F. amylovorans*	mar	FL	seawater	C	gl	0	2	0	2	0	0
Flavobacterium	*F. aquatile*	clin, mar, fre, ter	FL, S, P	deep well, freshwater, freshwater sediments, freshwater microbial mats, surface of freshwater animals, wastewater, soil, rhizosphere, gut of the earthworm, insects, sea ice, glacier ice, maine sediment, marine algae, clinical animal speciments of fish	C, F+	d	35	84	3	2	3	1
Formosa	*F. algae*	mar	S	marine green and brown algae, sponge	C	gl	2	3	0	0.5	2.5	0
Fulvibacter	*F. tottoriensis*	mar	FL	sediment	C		0	1	1	0	0	0
Gaetbulibacter	*G.saemankumensis*	mar	FL	tidal flat sediment (Korean: gaetbul), coastal seawater	F-	gl	1	4	2	2	0	0
Gaetbulimicrobium	*G. brevivitae*	⟶ *Aquimarina brevivitae*					1	0				
Galbibacter	*G. mesophilus*	mar	FL	sediment	C		0	1	1	0	0	0
Gangjinia	*G. marincola*	mar	FL	coastal seawater	C		0	1	0	1	0	0
Gelidibacter	*G. algens*	mar	FL, S	sea ice core, Antarctic lacustrine, sea-ice algae, Mediterranean sea water	C	gl	4	4	0	2	2	0
Gillisia	*G. limnaea*	mar	FL, S	microbial mats, sea-ice algae, marine sponge, seawater	F-		5	6	0	1	5	0
Gilvibacter	*G. sediminis*	mar	FL	sediment	C		0	1	1	0	0	0
Gramella	*G. echinicola*	mar	FL, S	sea urchin, marine sediment, tidal flat	C	gl	2	4	2	0	2	0
Hyunsoonleella	*H. jejuensis*	mar	FL	seawater	C		0	1	0	1	0	0
Jejuia	*J. pallidilutea*	mar	FL	seawater	F-		0	1	0	1	0	0
Joostella	*J. marina*	mar	FL	seawater	F-		0	1	0	1	0	0
Kaistella	*K. koreensis*	⟶ *Chryseobacterium koreense*					1	0				
Kordia	*K. algicida*	mar	FL, S	sea water sample associated with red tide	C		1	2	0	1	1	0
Kriegella	*K. aquimaris*	mar	FL, S	seawater	F+	gl	0	1	0	1	0	0
Krokinobacter	*K. genikus*	mar	FL, S	sediment	C	nd	3	3	3	0	0	0
Lacinutrix	*L. copepodicola*	mar	S	calanoid copepod, marine red algae	F-		1	3	0	0	3	0

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Table 2.S2 (continued)

Genus	Type strain	Envir.	Rel.	Isolation source	Pig.	Gl.	2006	2012	Sed	SW	S	P
Leeuwenhoekiella	*L. marinoflava*	mar	FL, S	Antarctic, North Sea off Aberdeen, Mediterranean Sea seawater, sea urchin, coral	F-	gl	2	4	0	2	2	0
Leptobacterium	*L. flavescens*	mar	S	sponge *Clathria* (*Microciona*) *eurypa*	F-		0	1	0	0	1	0
Lutaonella	*L. thermophila*	mar	FL	water of a coastal hot spring	F-		0	1	0	1	0	0
Lutibacter	*L. litoralis*	mar	FL	tidal flat sediment	C		0	4	4	0	0	0
Lutimonas	*L. vermicola*	mar	S	marine polychaete *Periserrula leucophryna*	C		0	1	0	0	1	0
Maribacter	*M. sedimenticola*	mar	FL, S	Arctic marine sediment, Antarctic green alga, red alga, sea water, North Sea water (Helgoland)	F-	gl	5	10	2	4	4	0
Mariniflexile	*M. gromovii*	mar	FL, S	seawater, sea urchin, alginate-extraction plant	F-	gl	0	3	0	1	2	0
Maritimimonas	*M. rapanae*	mar	S	veined rapa whelk (*Rapana venosa*)	F-		0	1	0	0	1	0
Marixanthomonas	*M. ophiurae*	mar	S	deep-sea brittle star	C		0	1	0	0	1	0
Meridianimaribacter	*M. flavus*	mar	FL	sandy sediment	F-	gl	0	1	1	0	0	0
Mesoflavibacter	*M. zeaxanthinifaciens*	mar	FL	seawater	C	gl	0	1	0	1	0	0
Mesonia	*M. algae*	mar	FL, S	seawater, green alga, seaweed	F-		1	3	0	1	2	0
Muricauda	*M. ruestringensis*	mar	FL	tidal flat sediment, salt lake near Hwajinpo Beach, coastal hot spring	F-		3	7	3	4	0	0
Muriicola	*M. jejuensis*	mar	FL	seawater	C		0	1	0	1	0	0
Myroides	*M. odoratus*	cli, mar	FL, S, P	human clinical specimens, seawater, deep-sea sediment	F-		2	6	1	2	0	0
Nonlabens	*N. tegetincola*	mar, fre	FL	microbial mat, seawater, sediment, subtropical estuary, faeces of the mollusc	C, F+	d	1	7	3	1	1	0
Olleya	*O. marilimosa*	mar	FL, S	particulate material of seawater	F-	gl	1	2	0	1	1	0
Ornithobacterium	*O. rhinotracheale*	cli	P, S	avian respiratory tract	P-		1	1				
Persicivirga	*P. xylanidelens*	⟶ *Nonlabens* sp.					1	0				
Pibocella	*P. ponti*	mar	S	green alga *Acrosiphonia sonderi*	F-	gl	0	1	0	0	1	0
Planobacterium	*P. taklimakanense*	ter	FL	desert soil	F-		0	1				
Polaribacter	*P. filamentus*	mar	FL	surface seawater Gangjin bay, Korea; Sea of Japan, Russia; Dokdo, Korea, seawater in pack ice, Deadhorse Alaska, sea ice, Antarctica	C	d	4	7	0	7	0	0
Pontirhabdus	*P. pectinivorans*	mar	FL	seawater	C	gl	0	1	0	1	0	0
Pseudozobellia	*P. thermophila*	mar	S	green alga *Ulva fenestrata*	F+	gl	0	1	0	0	1	0
Psychroflexus	*P. torquis*	mar	FL	Antarctic sea ice, marine solar saltern, hypersaline lake	C	d	3	5	1	4	0	0
Psychroserpens	*P. burtonensis*	mar	FL, S	seashore at Gangneung, Korea; Antarctic lacustrine and sea ice habitats	C		1	2	0	0	1	0

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Table 2.S2 (continued)

Genus	Type strain	Envir.	Rel.	Isolation source	Pig.	Gl.	2006	2012	Sed	SW	S	P
Riemerella	*R. anatipestifer*	cli	P	ducks, geese, turkeys, and waterfowl with septicemic disease	P-		2	2				
Robiginitalea	*R. biformata*	mar	FL	seawater Sargasso Sea, Atlantic Ocean, marine sediment	C		1	2	1	1	0	0
Salegentibacter	*S. salegens*	mar, ter	FL, S	hypersaline lakes, sponge, holothurian, sea urchin, marine sediment, marine solar saltern	C		3	7	1	2	3	0
Salinimicrobium	*S. catena*	mar, ter	FL	salt lake and soil, sediment and tidal flat sediment	C	d	0	5	3	0	0	0
Sandarakinotalea	*S. sediminis*	⟶ *Nonlabens sediminis*					1	0				
Sediminibacter	*S. furfurosus*	mar	FL	sediment	P-		0	1	1	0	0	0
Sediminicola	*S. luteus*	mar	FL	sediment	C		0	1	1	0	0	0
Sejongia	*S. antarctica*	⟶ *Chryseobacterium antarcticum*					2	0				
Sinomicrobium	*S. oceani*	mar	FL	sediment	nd	nd	0	1	1	0	0	0
Snuella	*S. lapsa*	mar	FL	tidal flat sediment	F-	gl	0	1	1	0	0	0
Soonwooa	*S. buanensis*	mar	FL	seawater	C		0	1	0	1	0	0
Spongiibacterium	*S. flavum*	mar	S	marine sponge *Halichondria oshoro*	F-		0	1	0	0	1	0
Stanierella	*S. latercula*	⟶ *Aquimarina latercula*					1	0				
Stenothermobacter	*S. spongiae*	⟶ *Nonlabens spongiae*					1	0				
Subsaxibacter	*S. broadyi*	mar	FL, S	cyanobacterial biofilms attached to the undersides of partially buried quartz stones	F-	gl	1	1	0	0	1	0
Subsaximicrobium	*S. wynnwilliamsii*	mar	FL, S	cyanobacterial biofilms attached to the undersides of partially buried quartz stones	F-	gl	2	2	2	0	0	0
Tamlana	*T. crocina*	mar	FL	beach sediment, seawater	C		0	2	1	1	0	0
Tenacibaculum	*T. maritimum*	mar	FL, S, P	diseased red sea bream Fingerling and sole, sole, sponge, turbot, sea bass, bryozoan, sea anemone, green alga, Pacific oyster, epiflora of halibut eggs, tidal flat sediment, coastal seawater	C, F-	gl	6	18	3	3	11	1
Ulvibacter	*U. litoralis*	mar	FL, S	green alga, Antarctic coastal seawater	F+	d	1	2	0	1	1	0
Vitellibacter	*V. vladivostokensis*	mar	FL, S	holothurian, tidal-flat sediment	F+		1	2	1	0	1	0
Wautersiella	*W. falsenii*	cli	P, S	surgical wound, clinical laboratories in Belgium	nd	nd	0	1				
Weeksella	*W. virosa*	cli	P, S	human clinical specimens	nd		1	1				
Winogradskyella	*W. thalassocola*	mar	FL, S	green and brown alga, sponge, marine sediments, seawater, sea urchin, starfish	F-	gl	4	10	1	3	6	0
Yeosuana	*Y. aromativorans*	ter, fre	FL	aesturine sediment, benzo[a]pyrene (BaP) and pyrene enrichment culture	C		0	1				
Zeaxanthinibacter	*Z. enoshimensis*	mar	FL	seawater	C	gl	0	1	0	1	0	0
Zhouia	*Z. amylolytica*	mar	FL	sediment	nd		0	1	1	0	0	0

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Table 2.S2 (continued)

Genus	Type strain	Envir.	Rel.	Isolation source	Pig.	Gl.	2006	2012	Sed	SW	S	P
Zobellia	*Z. galactanovorans*	mar	FL, S	brown, green and red alga, surface sediment, seawater	F+	gl	5	5	1	1	3	0
Zunongwangia	*Z. profunda*	mar	FL	deep-sea sediment	F-		0	1	1	0	0	0

Table 2.S3 Strains of this study within the genera and the 16S rRNA sequence identity with the next relative type strain (in %). The strains were subsequently isolated on HaHa agar, 2216E agar or SYL agar (ARA arabinose, CAA casamino acids, CEL cellobiose, GAL galactose, GLU glucose, MAL malate, NAG N-acetylglucosamine, RAM rhamnose, XYL xylose) and physiological characteristics were observed (KanaR, kanamycin resistence; F, flexirubin type pigments; Ir, iridescence; gl, gliding motility; ly, agar lysis). All strains isolated on HaHa medium or enriched (enrich) were initially identified as *Flavobacteriaceae* by PCR screen. ANT, Antarctica; PO, Pacific Ocean; HK, Hong Kong; GER, Germany; DK, Denmark; KR, Korea

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Maribacter dokdonensis									
***M. dokdonensis* DSW-8 (>98.7%)**				**-**	**Ir**	**gl**	**ly**	**surface seawater**	**Dokdo Island, KR**
MAR_2009_71	plating	NAG		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_74	plating	GLU		-				20 μm phytoplank.	Sylt, List
MAR_2009_221	plating	CEL		-		gl	ly	80 μm phytoplank.	Sylt, List
RHA_13	plating	2216E		-				Starfish	Sylt, List, Beach
RHA_53	plating	2216E		-				mussel surface	Sylt, List, Beach
RHA_58	plating	CAA		-				diatomes	Sylt, List, Beach
RHA_60	plating	2216E		-				mussel surface	Sylt, List, Beach
RHA_67	plating	CEL		-				*Ulva lactuca*	Sylt, List, Beach
RHA_81	plating	CAA		-		gl		diatomes	Sylt, List, Beach
RHA_95	plating	CAA		-	Ir	gl		diatomes	Sylt, List, Beach
RHA_112	plating	2216E		-				*Ulva lactuca*	Sylt, List, Beach
SRO_19	plating	RAM		-	Ir		ly	intertidal sediment	Sylt, Königshafen
SRO_21	plating	XYL		-				intertidal sediment	Sylt, Königshafen
SRO_22	plating	GAL		-				intertidal sediment	Sylt, Königshafen
SRO_24	plating	RAM		-		gl	ly	intertidal sediment	Sylt, Königshafen
SRO_314	plating	RAM		-				intertidal sediment	Sylt, Königshafen
SRO_351	plating	XYL		-				intertidal sediment	Sylt, Hausstrand
SRO_411	plating	GAL		-				intertidal sediment	Sylt, Hausstrand
SRO_470	plating	GAL		-		gl	ly	intertidal sediment	Sylt, Hausstrand
SRO_476	plating	XYL		-				intertidal sediment	Sylt, Hausstrand
TBL_15	plating	CAA		-		gl	ly	intertidal sediment	Sylt, Hausstrand
TBL_23	plating	NAG		-		gl	ly	intertidal sediment	Janssand, MF
TBL_41	plating	CEL		-		gl	ly	intertidal sediment	Janssand, UF
TBL_78_130	plating	NAG	k	-				intertidal sediment	Sylt, Königshafen
Maribacter stanieri									
***M. stanieri* KMM 6046 (>99.1%)**				**-**	**-**	**gl**	**-**	***Ulva***	**Dokdo Island, KR**
Hel_I_7	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_14	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_22	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_23	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_25	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_27	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_54	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_57	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_82	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_87	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Hel_I_95	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
***Maribacter* sp. I**									
***M. sedimenticola* CCUG 47098 (95-97%)**				**-**	**-**	**-**	**ly**	**bottom sediment**	**Dokdo Island, KR**
MAR_2009_60	plating	NAG		-				20 μm phytoplank.	Sylt, List
MGE_SAT_358	enrich	2216E		-				intertidal sediment	Harlesiel
Maribacter sedimenticola									
***M. sedimenticola* CCUG 47098 (>99.3%)**				**-**	**-**	**-**	**ly**	**bottom sediment**	**Dokdo Island, KR**
MAR_2009_72	plating	GLU		-				20 μm phytoplank.	Sylt, List
SRO_238	plating	RAM		-				intertidal sediment	Sylt, Königshafen
SRO_412	plating	XYL		-				intertidal sediment	Sylt, Königshafen

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Maribacter forsetii									
***M. forsetii* KT02ds18-6 (>98.7%)**				**-**	**-**	**gl**	**-**	**surface seawater**	**Helgoland Island, GER**
MAR_2009_128	plating	NAG		-				20 μm phytoplank.	Sylt, List
SRO_1	plating	2216E		-				*Fucus ceranoides*	Sylt, List
SRO_12	plating	2216E		-				*Fucus ceranoides*	Sylt, List
SRO_10	plating	2216E		-				intertidal sediment	Sylt, List
MAR_2009_297	plating	CAA		-				Jellyfish	Sylt, List, Beach
RHA_73	plating	CAA		-				crab surface	Sylt, List, Beach
SRO_138	plating	2216E		-				intertidal sediment	Sylt, West beach
SRO_381	plating	RAM		-				intertidal sediment	Sylt, Königshafen
SRO_25	plating	XYL		-		gl	ly	intertidal sediment	Sylt, Hausstrand
SRO_26	plating	XYL		-		gl	ly	intertidal sediment	Sylt, Hausstrand
TBL_26	plating	MAL		-		gl	ly	intertidal sediment	Sylt, Hausstrand
***Maribacter* sp. II**									
***M. forsetii* KT02ds18-6 (95-97%)**				**-**	**-**	**gl**	**-**	**surface seawater**	**Helgoland Island, GER**
TBL_80	plating	ARA		-				intertidal sediment	Sylt, Königshafen
TBL_87_140	plating	MAL		-				intertidal sediment	Sylt, Königshafen
TBL_105	plating	CAA		-				intertidal sediment	Sylt, Königshafen
TBL_20	plating	NAG		-		gl	ly	intertidal sediment	Janssand, MF
***Maribacter* sp. III**									
***M. sedimenticola* CCUG 47098 (97%)**				**-**	**-**	**-**	**ly**	**bottom sediment**	**Dokdo Island, KR**
TBL_101_154	plating	ARA		-				intertidal sediment	Sylt, Königshafen
***Maribacter* sp. IV**									
***M. antarcticus* CL-AP4 (98%)**				**-**	**-**	**-**	**-**	***Pyramimonas***	**Southern Ocean, ANT**
MGE_SAT_274	enrichm	2216E		-				intertidal sediment	Harlesiel
Zobellia russellii									
***Z. russellii* KMM 3677 (>99.7%)**				**+**	**-**	**gl**	**ly**	***Acrosiphonia***	**Troitsa Bay, KR**
MAR_2009_226	plating	NAG		+				80 μm phytoplank.	Sylt, List
RHA_17	plating	2216E	k	+				20 μm phytoplank.	Sylt, List
RHA_40	plating	CAA		+		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_61	plating	2216E		+				Starfish	Sylt, List, Beach
RHA_66	plating	CEL		+				*Ulva lactuca*	Sylt, List, Beach
RHA_85	plating	MAL		+		gl		*Ulva lactuca*	Sylt, List, Beach
MAR_2009_119	plating	NAG		+		gl		intertidal sediment	Sylt, Königshafen
MAR_2009_120	plating	NAG		+				intertidal sediment	Sylt, Königshafen
MAR_2009_186	plating	CEL		+		gl		intertidal sediment	Sylt, Königshafen
MAR_2009_168	plating	NAG		+				clam byssus thread	Sylt, Königshafen
TBL_37	plating	CEL		+		gl	ly	intertidal sediment	Sylt, Königshafen
TBL_12	plating	CAA		+		gl	ly	intertidal sediment	Sylt, Hausstrand
TBL_21	plating	NAG		+		gl	ly	intertidal sediment	Janssand, MF
Zobellia amurskyensis									
***Z. amurskyensis* KMM 3526 (>99.4%)**				**+**	**-**	**gl**	**ly**	**surface seawater**	**Amursky Bay, KR**
MAR_2009_230	plating	CEL		+		gl		80 μm phytoplank.	Sylt, List
SRO_20	plating	RAM		+				intertidal sediment	Sylt, List
TBL_82_395	plating	ARA	k	+				intertidal sediment	Sylt, Königshafen
TBL_85_137	plating	MAL	k	+				intertidal sediment	Sylt, Königshafen
TBL_88	plating	CAA		+				intertidal sediment	Sylt, Königshafen
TBL_104	plating	CAA	k	+				intertidal sediment	Sylt, Königshafen
TBL_113	plating	MAL		+				intertidal sediment	Sylt, Königshafen
MGE_SAT_695_2	enrichm	2216E	k	+				intertidal sediment	Harlesiel
TBL_90	plating	NAG	k	+				intertidal sediment	Janssand,UF
Zobellia amurskyensis									
***Z. uliginosa* ZoBell 553 (>98.9%)**				**+**	**-**	**gl**	**ly**	**surface sediment**	**Limon, Costa Rica**
MAR_2009_138	plating	CEL		+		gl		20 μm phytoplank.	Sylt, List
TBL_19	plating	NAG		+		gl	ly	intertidal sediment	Sylt, Königshafen
TBL_79	plating	ARA		+				intertidal sediment	Sylt, Königshafen
TBL_103	plating	ARA	k	+				intertidal sediment	Sylt, Königshafen
TBL_110	plating	CAA	k	+				intertidal sediment	Sylt, Königshafen

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Kriegella aquimaris									
***K. aquimaris* KMM 3665 (>98.7%)**				+	-	gl	-	**surface seawater**	**Amursky Bay, KR**
TBL_112_391	plating	MAL	k	+				intertidal sediment	Sylt, Königshafen
TBL_69	plating	MAL		+				intertidal sediment	Sylt, Hausstrand
Muricauda ruestringensis									
***M. ruestringensis* DSM 13258 (>99%)**				-	-	-	-	**intertidal sediment**	**Jadebusen Bay, GER**
MAR_2010_74	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_124	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_125	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_133	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_216	96 pin	HaHa		-				surface seawater	Sylt, List
RHA_87	plating	NAG	k	-				20 μm phytoplank.	Sylt, List
RHA_88	plating	NAG	k	-				20 μm phytoplank.	Sylt, List
RHA_111	plating	2216E		-				20 μm phytoplank.	Sylt, List
MAR_2009_44	plating	GLU		-		gl		intertidal sediment	Sylt, Königshafen
MAR_2009_167	plating	GLU		-				intertidal sediment	Sylt, Königshafen
***Muricauda* sp. I**									
***M. flavescens* SW-62 (96.3%)**				-	Ir	-	-	**surface seawater**	**Hwajinpo Beach, KR**
MAR_2010_75	96 pin	HaHa		-				surface seawater	Sylt, List
Arenibacter troitsensis									
***A. troitsensis* CM 11736 (>99.3%)**				-	-	-	-	**bottom sediment**	**Troitsa Bay, KR**
RHA_47	plating	2216E		-				mussel surface	Sylt, List, Beach
RHA_82	plating	2216E		-				mussel surface	Sylt, List, Beach
MAR_2010_101	96 pin	HaHa		+				sediment porewater	Sylt, West beach
TBL_36	plating	CEL		-				intertidal sediment	Sylt, Königshafen
TBL_47	plating	CEL		-				intertidal sediment	Sylt, Königshafen
TBL_83_135	plating	MAL	k	-				intertidal sediment	Sylt, Königshafen
TBL_84_136	plating	MAL		-				intertidal sediment	Sylt, Königshafen
TBL_86_138	plating	CAA		-				intertidal sediment	Sylt, Königshafen
SRO_232	plating	RAM		-				intertidal sediment	Sylt, Königshafen
TBL_48	plating	CAA		-				intertidal sediment	Sylt, Hausstrand
TBL_56	plating	CAA		-				intertidal sediment	Sylt, Hausstrand
MGE_SAT_544_1	enrich	2216E		+				intertidal sediment	Harlesiel
TBL_35	plating	NAG		-				intertidal sediment	Janssand, UF
TBL_39	plating	CAA		-				intertidal sediment	Janssand, UF
TBL_45	plating	CEL		-				intertidal sediment	Janssand, UF
TBL_52	plating	CEL		-				intertidal sediment	Janssand, UF
TBL_75_126	plating	MAL		-				intertidal sediment	Janssand, UF
Arenibacter palladensis									
***A. palladensis* CIP 108849 (>99.3%)**				-	-	gl	-	***Ulva***	**Pallada Bay, KR**
MAR_2009_79	plating	GLU		-				20 μm phytoplank.	Sylt, List
SRO_172	plating	XYL		-				intertidal sediment	Sylt, Königshafen
SRO_174	plating	XYL		-				intertidal sediment	Sylt, Königshafen
SRO_198	plating	GAL		-				intertidal sediment	Sylt, Hausstrand
SRO_240	plating	RAM		-				intertidal sediment	Sylt, Hausstrand
Arenibacter echinorum									
***A. echinorum* KMM 6032 (>98.9%)**				-	-	gl	-	***Strongylocentrotus***	**Troitsa Bay, KR**
SRO_243	plating	RAM		-				intertidal sediment	Sylt, Hausstrand
SRO_210	plating	XYL		-				intertidal sediment	Janssand, MF
SRO_393	plating	XYL		-				intertidal sediment	Janssand, MF
***Arenibacter* sp. I**									
***A. nanhaiticus* NH36A (93.7–94.8%)**				-	Ir	gl	-	**sediment**	**South China Sea**
SRO_5	plating	2216E		-				intertidal sediment	Sylt, Weststrand
SRO_202	plating	2216E		-				intertidal sediment	Sylt, Weststrand
SRO_310	plating	XYL		-				intertidal sediment	Sylt, Hausstrand
SRO_366	plating	GAL		-				intertidal sediment	Sylt, Hausstrand

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
***Arenibacter* sp. II**									
***A. echinorum* KMM 6032 (94.5–97.5%)**				**-**	**-**	**gl**	**-**	***Strongylocentrotus***	**Troitsa Bay, KR**
SRO_28	plating	2216E		-				intertidal sediment	Sylt, Weststrand
SRO_242	plating	RAM		-				intertidal sediment	Sylt, Hausstrand
Zeaxanthinibacter enoshimensis									
***Z. enoshimensis* TD-ZE3 (>99.1%)**				**-**	**-**	**gl**	**-**	**surface seawater**	**Enoshima Island, Japan**
MAR_2010_32	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_153	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
MAR_2010_194	96 pin	HaHa		-				sediment porewater	Sylt, West beach
Cellulophaga lytica									
***C. lytica* ATCC 23178 (>99.7%)**				**-**	**Ir**	**gl**	**ly**	**beach mud**	**Limon, Costa Rica**
MAR_2009_61	plating	NAG		-				20 μm phytoplank.	Sylt, List
MAR_2009_66	plating	NAG		-				20 μm phytoplank.	Sylt, List
MAR_2009_69	plating	GLU		-				20 μm phytoplank.	Sylt, List
MAR_2009_70	plating	GLU		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_73	plating	NAG		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_77	plating	GLU		-				20 μm phytoplank.	Sylt, List
MAR_2009_80	plating	GLU		-				20 μm phytoplank.	Sylt, List
MAR_2009_136	plating	CEL		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_139	plating	CEL		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_202	plating	CEL		-				20 μm phytoplank.	Sylt, List
MAR_2009_203	plating	CEL		-				20 μm phytoplank.	Sylt, List
RHA_42	plating	2216E	k	-		gl		20 μm phytoplank.	Sylt, List
RHA_43	plating	2216E	k	-		gl		20 μm phytoplank.	Sylt, List
RHA_44	plating	2216E	k	-		gl		20 μm phytoplank.	Sylt, List
RHA_51	plating	2216E	k	-		gl		20 μm phytoplank.	Sylt, List
RHA_52	plating	2216E	k	-		gl		20 μm phytoplank.	Sylt, List
RHA_62	plating	NAG		-				20 μm phytoplank.	Sylt, List
MAR_2009_161	plating	MAL		-		gl		80 μm phytoplank.	Sylt, List
MAR_2009_163	plating	MAL		-				80 μm phytoplank.	Sylt, List
MAR_2009_222	plating	CEL		-		gl	ly	80 μm phytoplank.	Sylt, List
RHA_27	plating	CAA		-	Ir	gl		Crab surface	Sylt, List, Beach
RHA_28	plating	CAA		-	Ir	gl		Crab surface	Sylt, List, Beach
RHA_29	plating	CAA		-	Ir	gl		Crab surface	Sylt, List, Beach
RHA_30	plating	CAA		-		gl		Crab surface	Sylt, List, Beach
RHA_80	plating	CAA		-				Crab surface	Sylt, List, Beach
RHA_84	plating	2216E		-				Lanice surface	Sylt, List, Beach
RHA_70	plating	CAA		-				Crab surface	Sylt, List, Beach
RHA_21	plating	2216E		-				Starfish surface	Sylt, List, Beach
RHA_14	plating	2216E		-	Ir	gl		*Fucus ceranoides*	Sylt, List, Beach
RHA_5	plating	CAA		-	Ir	gl		*Polysiphonia lanosa*	Sylt, List, Beach
RHA_6	plating	CAA		-		gl		*Polysiphonia lanosa*	Sylt, List, Beach
RHA_35	plating	CAA		-		gl		*Polysiphonia lanosa*	Sylt, List, Beach
RHA_22	plating	CAA		-	Ir			*Ulva lactuca*	Sylt, List, Beach
RHA_24	plating	CAA		-	Ir	gl		*Ulva lactuca*	Sylt, List, Beach
RHA_36	plating	CAA		-	Ir	gl		*Ulva lactuca*	Sylt, List, Beach
RHA_37	plating	CAA		-		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_38	plating	MAL		-		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_39	plating	MAL		-		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_64	plating	CEL		-				*Ulva lactuca*	Sylt, List, Beach
RHA_65	plating	CEL		-		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_79	plating	CEL		-	Ir	gl		*Ulva lactuca*	Sylt, List, Beach
TBL_76	plating	MAL	k	-				intertidal sediment	Janssand, UF
SRO_27	plating	GAL		-				intertidal sediment	Janssand, MF
TBL_16	plating	NAG		-			ly	intertidal sediment	Sylt, Hausstrand
RHA_31	plating	MAL		-		gl		intertidal sediment	Sylt, Hausstrand
RHA_32	plating	MAL		-		gl		intertidal sediment	Sylt, Hausstrand
RHA_33	plating	MAL		-	Ir	gl		intertidal sediment	Sylt, Hausstrand
RHA_34	plating	MAL		-	Ir	gl		intertidal sediment	Sylt, Hausstrand
TBL_17	plating	NAG		-			ly	intertidal sediment	Sylt, Königshafen

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Cellulophaga sp. I									
***C. lytica* ATCC 23178 (98.7%)**				**-**	**Ir**	**gl**	**ly**	**beach mud**	**Limon, Costa Rica**
RHA_19	plating	CAA		-	Ir	gl		*Polysiphonia lanosa*	Sylt, List, Beach
RHA_20	plating	2216E		-				Starfish surface	Sylt, List, Beach
***Cellulophaga* sp. II**									
***C. fucicola* NN015860 (98.0–98.6%)**				**-**	**Ir**	**gl**	**ly**	***Fucus***	**Hirsholm Island, DK**
MGE_SAT_694	enrich	2216E	k	-				intertidal sediment	Harlesiel
Cellulophaga baltica									
***C. baltica* NN015840 (99.8%)**				**-**	**Ir**	**gl**	**ly**	***Fucus***	**Bornholm Island, DK**
MAR_2009_160_2	plating	NAG		-				Crab surface	Sylt, List, Beach
Cellulophaga pacifica									
***C. pacifica* KMM 3664 (>99.7%)**				**-**	**Ir**	**gl**	**ly**	**surface seawater**	**Amursky Bay, KR**
MAR_2010_47	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_197	96 pin	HaHa		-				sediment porewater	Sylt, West beach
***Cellulophaga* sp. III**									
***C. pacifica* KMM 3664 (>99.7%)**				**-**	**Ir**	**gl**	**ly**	**surface seawater**	**Amursky Bay, KR**
Hel_I_12	96 pin	HaHa		-				intertidal sediment	Helgoland, Kabeltonne
Sediminicola luteus									
***S. luteus* CNI-3 (>98.9%)**				**-**	**-**	**-**	**-**	**marine sediment**	**Sea of Japan, PO**
MAR_2010_47	98 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_167	98 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_181	98 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_189	98 pin	HaHa		-				sediment porewater	Sylt, West beach
MGE_SAT_103	enrich	2216E		-				intertidal sediment	Harlesiel
MGE_SAT_710	enrich	2216E	k	-				intertidal sediment	Harlesiel
***Sediminicola* sp. I**									
***S. luteus* CNI-3 (98.4%)**				**-**	**-**	**-**	**-**	**marine sediment**	**Sea of Japan, PO**
MAR_2010_190	98 pin	HaHa		-				sediment porewater	Sylt, West beach
***Leeuwenhoekiella* sp. I**									
***L. aequorea* CCUG 50091 (95.7–98.5%)**				**-**	**-**	**gl**	**-**	**surface seawater**	**Gunnerus Ridge, ANT**
MAR_2009_132	plating	CEL		-				20 μm phytoplank.	Sylt, List
SRO_2	plating	2216E		-				intertidal sediment	List, Sylt
SRO_3	plating	2216E		-		gl		intertidal sediment	List, Sylt
***Leeuwenhoekiella* sp. II**									
***L. aequorea* CCUG 50091 (96.8–97.5%)**				**-**	**-**	**gl**	**-**	**surface seawater**	**Gunnerus Ridge, ANT**
MAR_2010_192	96 pin	HaHa		-				sediment porewater	Sylt, West beach
Gramella gaetbulicola									
***G. gaetbulicola* RA5-111 (>98.5%)**				**-**	**-**	**gl**	**-**	**intertidal sediment**	**Jeonbuk, KR**
MAR_2010_82	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_83	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_91	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_109	96 pin	HaHa		-				sediment porewater	Sylt, West beach
SRO_17	plating	2216E		-				intertidal sediment	Sylt, West beach
SRO_287	plating	RAM		-				intertidal sediment	Sylt, Hausstrand
TBL_102	plating	ARA	k	-				intertidal sediment	Sylt, Königshafen
MGE_SAT_800	enrich	2216E	k	-				intertidal sediment	Harlesiel
Gramella marina									
***G. marina* KMM 6048 (>98.7%)**				**-**	**-**	**gl**	**-**	***Strongylocentrotus***	**Troitsa Bay, KR**
MAR_2010_21	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
TBL_53	plating	NAG		-				intertidal sediment	Sylt, Hausstrand
TBL_99	plating	NAG		-				intertidal sediment	Sylt, Königshafen
MGE_SSAT_816	enrich	CAA	k	-				intertidal sediment	Harlesiel
TBL_38	plating	CEL		-				intertidal sediment	Janssand, MF
TBL_49	plating	CAA		-				intertidal sediment	Janssand, UF
TBL_70	plating	MAL		-				intertidal sediment	Janssand, UF
TBL_73	plating	MAL		-				intertidal sediment	Janssand, UF

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Gramella echinicola									
***G. echinicola* JCM 13510 (>99.9%)**				**-**	**-**	**gl**	**-**	***Strongylocentrotus***	**Troitsa Bay, KR**
MAR_2010_2	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_14	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_85	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_88	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_92	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_96	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_141	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_142	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_156	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
MAR_2010_157	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_164	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_195	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_198	96 pin	HaHa		-				sediment porewater	Sylt, West beach
'*Gramella forsetii*'									
'*G. forsetii*' KT0803 (>98.9%)				**-**	**-**	**gl**	**-**	**surface seawater**	**Helgoland Island, GER**
MAR_2010_87	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_147	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_163	96 pin	HaHa		-				sediment porewater	Sylt, West beach
Hel_I_64	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Gramella portivictoriae									
***G. portivictoriae* UST040801-001 (>99.0%)**				**-**	**-**	**gl**	**-**	**marine sediment**	**Victoria Harbour, HK**
MAR_2010_213	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_25	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_81	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_84	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_103	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_110	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_143	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
MAR_2010_155	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_166	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_200	96 pin	HaHa		-				sediment porewater	Sylt, West beach
TBL_95	plating	NAG		-				intertidal sediment	Sylt, Königshafen
TBL_96	plating	NAG		-				intertidal sediment	Sylt, Königshafen
MGE_SSAT_702	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SSAT_817	enrich	CAA	k	-				intertidal sediment	Harlesiel
MGE_SSAT_818	enrich	CAA	k	-				intertidal sediment	Harlesiel
Hel_I_59	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Gramella* sp. I**									
***G. portivictoriae* UST040801-001 (97.1%)**				**-**	**-**	**gl**	**-**	**marine sediment**	**Victoria Harbour, HK**
MAR_2010_102	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
Saligentibacter mishustinae									
***S. mishustinae* KCTC 12263 (99.7%)**				**-**	**-**	**-**	**-**	***Strongylocentrotus***	**Troitsa Bay, KR**
TBL_100	plating	ARA	k	-				intertidal sediment	Sylt, Königshafen
Saligentibacter salarius									
***S. salarius* ISL-6 (99.1%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Yellow Sea, Korea**
Hel_I_34	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Saligentibacter* sp. I**									
***S. salegens* ACAM 48 (>98.8%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Vestfold Hills, East ANT**
MGE_SAT_704	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_706	enrich	2216E	k	-				intertidal sediment	Harlesiel
***Saligentibacter* sp. II**									
***S. salegens* ACAM 48 (>98.7%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Vestfold Hills, East ANT**
Hel_I_6	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_16	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Mesonia algae									
***M. algae* CCUG 47092 (100%)**				**-**	**-**	**-**	**-**	***Acrosiphonia***	**Troitsa Bay, KR**
RHA_77	plating	2216E	k	-				20 μm phytoplank.	Sylt, List
Lacinutrix copepodicola									
***L. copepodicola* DJ3 (98.9%)**				**-**	**-**	**-**	**-**	***Paralabidocera***	**Ace Lake, East ANT**
MGE_SAT_368	enrich	2216E		+				intertidal sediment	Harlesiel
***Lacinutrix* sp. I**									
***L. algicola* AKS432 (96.5%)**				**-**	**-**	**-**	**-**	**red alga**	**King George Island, ANT**
Hel_I_90	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Gillisia mitskevichiae									
***G. mitskevichiae* KCTC 12261 (>99.4%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Amursky Bay, KR**
MAR_2010_171	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
MAR_2010_182	96 pin	HaHa		-		gl		sediment porewater	Sylt, West beach
Gillisia myxillae									
***G. myxillae* UST050418-085 (1000%)**				**-**	**-**	**-**	**-**	***Myxilla***	**Friday Harbor, USA**
Hel_I_29	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_41	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Gillisia* sp. I**									
***G. mitskevichiae* KCTC 12261 (97.9–98.1%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Amursky Bay, KR**
Hel_I_11	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_18	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_19	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_86	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Zunongwangia profunda									
***Z. profunda* SMA-97 (>99.9%)**				**-**	**-**	**-**	**-**	**deep-sea sediment**	**Okinawa Trough**
MAR_2010_43	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_48	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_51	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_57	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_65	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_67	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_122	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_126	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_127	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_128	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_129	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_134	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_135	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_204	96 pin	HaHa		-				surface seawater	Sylt, List
MAR_2010_219	96 pin	HaHa		-				surface seawater	Sylt, List
***Zunongwangia* sp. I**									
***Z. profunda* SMA-97 (96.7%)**				**-**	**-**	**-**	**-**	**deep-sea sediment**	**Okinawa Trough**
MAR_2010_100	96 pin	HaHa		-				surface seawater	Sylt, List
***Olleya* sp. I**									
***O. marilimosa* CAM030 (96.9–98.1%)**				**-**	**-**	**-**	**ly**	**surface seawater**	**Southern Ocean**
RHA_63	plating	CAA		-				*Ulva lactuca*	Sylt, List, Beach
RHA_69	plating	2216E		-				*Lanice*	Sylt, List, Beach
RHA_71	plating	2216E		-				*Lanice*	Sylt, List, Beach
MAR_2010_37	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MGE_SAT_332	enrich	2216E		-				intertidal sediment	Harlesiel
Hel_I_1	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_2	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_3	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_24	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_37	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_60	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_61	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_94	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne

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Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Lacinutrix copepodicola									
***L. copepodicola* DJ3 (98.9%)**				**-**	**-**	**-**	**-**	***Paralabidocera***	**Ace Lake, East ANT**
MGE_SAT_368	enrich	2216E		+				intertidal sediment	Harlesiel
***Lacinutrix* sp. I**									
***L. algicola* AKS432 (96.5%)**				**-**	**-**	**-**	**-**	**red alga**	**King George Island, ANT**
Hel_I_90	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Psychroserpens* sp. I**									
***P. burtonensis* ACAM 188 (95.3–95.9%)**				**-**	**-**	**-**	**-**	**Antarctic lacustrine**	**Burton Lake, ANT**
Hel_I_66	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Winogradskyella* sp. I**									
***W. rapida* SCB36 (98.6%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Scripps Pier, USA**
MGE_SAT_697	enrich	2216E	k	-				intertidal sediment	Harlesiel
***Winogradskyella* sp. II**									
***W. eximia* KMM 3944 (95.7%)**				**-**	**-**	**-**	**ly**	***Laminaria***	**G. of Peter the Great**
RHA_55	plating	2216E		-				mussel surface	Sylt, List, Beach
***Algibacter* sp. I**									
***A. lectus* DSM 15365 (96.4–98.6%)**				**-**	**-**	**gl**	**ly**	**green algae**	**G. of Peter the Great**
MGE_SAT_542	enrich	2216E		-				intertidal sediment	Harlesiel
RHA_19	plating	CAA		-	Ir	gl		*Polysiphonia lanosa*	Sylt, List, Beach
***Ulvibacter* sp. I**									
***U. antarcticus* IMCC3101 (96.4%)**				+	**-**	**-**	**-**	**surface seawater**	**King George Island, ANT**
MAR_2010_11	96 pin	HaHa		-				sediment porewater	Sylt, West beach
Aquimarina macrocephali									
***A. macrocephali* JAMB N27 (>99.9%)**				+	**-**	**gl**	**-**	**sediment**	**Kagoshima, Japan**
TBL_2	plating	MAL		-		gl		intertidal sediment	Sylt, Königshafen
TBL_55	plating	NAG		-		gl		intertidal sediment	Sylt, Königshafen
***Aquimarina* sp. I**									
***A. macrocephali* JAMB N27 (95.5–98.4%)**				+	**-**	**gl**	**-**	**sediment**	**Kagoshima, Japan**
MAR_2010_214	96 pin	HaHa		+				surface seawater	Sylt, List
MAR_2010_215	96 pin	HaHa		+				surface seawater	Sylt, List
***Aquimarina* sp. II**									
***A. addita* JAMB N27 (94.7–97.0%)**				**-**	**Ir**	**-**	**-**	**surface seawater**	**Jeju Island, KR**
SRO_221	plating	XYL		+				intertidal sediment	Sylt, Königshafen
TBL_9	plating	CAA		+		gl		intertidal sediment	Sylt, Königshafen
TBL_28	plating	MAL		+		gl		intertidal sediment	Janssand, UF
Krokinobacter eikastus									
***K. eikastus* PMA-26 (>99.9%)**				**-**	**-**	**-**	**-**	**sediment**	**Kisarazu, Japan**
SRO_11	plating	2216E		-		gl		*Fucus ceranoides*	Sylt, List
SRO_18	plating	2216E		-			ly	*Fucus ceranoides*	Sylt, List
Hel_I_63	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Krokinobacter* sp. I**									
***K. diaphorus* MSKK-32l (98.0%)**				**-**	**-**	**-**	**-**	**sediment**	**Kisarazu, Japan**
Hel_I_53	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
***Dokdonia/Krokinobacter* sp. II**									
***D. donghaensis* DSW-1 (97.1%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Dokdo Island, KR**
***K. genikus* Cos-13 (97.1%)**				**-**	**-**	**-**	**-**	**sediment**	**Odawara, Japan**
Hel_I_5	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_65	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Hel_I_91	96 pin	HaHa		-				surface seawater	Helgoland, Kabeltonne
Tenacibaculum gallaicum									
***T. gallaicum* A37.1 (>99.6%)**				**-**	**-**	**gl**	**-**	**sole culture**	**Galicia, Spain**
RHA_25	plating	CAA		-		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_26	plating	CAA		-		gl		*Ulva lactuca*	Sylt, List, Beach
RHA_16	plating	2216E	k	-				20 μm phytoplank.	Sylt, List
MAR_2009_227	plating	CEL		-				80 μm phytoplank.	Sylt, List

Continued on next page

Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	Kana^R	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
Tenacibaculum litoreum									
***T. litoreum* CL-TF13 (99.8%)**				**-**	**-**	**gl**	**-**	**intertidal sediment**	**Ganghwa Island, KR**
SRO_13	plating	2216E		-				intertidal sediment	Sylt, Westbeach
***Tenacibaculum* sp. I**									
***T. gallaicum* A37.1 (98.7%)**				**-**	**-**	**gl**	**-**	**sole culture**	**Galicia, Spain**
MGE_SAT_708	enrich	2216E	k	-				intertidal sediment	Harlesiel
***Tenacibaculum* sp. II**									
***T. mesophilum* MBIC1140 (93.3–95.8%)**				**-**	**-**	**gl**	**-**	***Halichondria***	**Numazu, Japan**
MAR_2009_124	plating	NAG		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_126	plating	NAG		-		gl		20 μm phytoplank.	Sylt, List
MAR_2009_134	plating	CEL		-				20 μm phytoplank.	Sylt, List
***Tenacibaculum* sp. III**									
***T. mesophilum* MBIC1140 (94.8–97.4%)**				**-**	**-**	**gl**	**-**	***Halichondria***	**Numazu, Japan**
MAR_2010_89	96 pin	HaHa		-				sediment porewater	Sylt, West beach
MAR_2010_175	96 pin	HaHa		-				sediment porewater	Sylt, West beach
***Tenacibaculum* sp. IV**									
***T. ovolyticum* EKD002 (97.8%)**				**-**	**-**	**gl**	**-**	**halibut eggs**	**Bergen, Norway**
MAR_2010_205	96 pin	HaHa		-				surface seawater	Sylt, List
***Tenacibaculum* sp. V**									
***T. ovolyticum* EKD002 (98.0%)**				**-**	**-**	**gl**	**-**	**halibut eggs**	**Bergen, Norway**
MAR_2010_191	96 pin	HaHa		-				sediment porewater	Sylt, West beach
***Polaribacter* sp. I**									
***P. butkevichii* KMM 3938 (97.1%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Amursky Bay, KR**
Hel_I_85	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
***Polaribacter* sp. II**									
***P. dokdonensis* DSW-5 (97.1%)**				**-**	**Ir**	**-**	**-**	**surface seawater**	**Dokdo Island, KR**
Hel_I_88	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
***Polaribacter* sp. III**									
***P. dokdonensis* DSW-5 (96.1%)**				**-**	**Ir**	**-**	**-**	**surface seawater**	**Dokdo Island, KR**
MAR_2010_29	96 pin	HaHa		-				sediment porewater	Sylt, West beach
Lutibacter litoralis									
***L. litoralis* CL-TF09 (>99.0%)**				**-**	**-**	**-**	**-**	**intertidal sediment**	**Ganghwa Island, KR**
MGE_SAT_468	enrich	2216E		-				intertidal sediment	Harlesiel
MGE_SAT_509_1	enrich	2216E		-				intertidal sediment	Harlesiel
MGE_SAT_686	enrich	2216E		-				intertidal sediment	Harlesiel
MGE_SAT_687	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_688	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_690_2	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_691	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_712	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_714	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_715	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_717	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_718	enrich	2216E		-				intertidal sediment	Harlesiel
MGE_SAT_719_2	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_717	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_720	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_782	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_788	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_791	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_794	enrich	2216E	k	-				intertidal sediment	Harlesiel
MGE_SAT_795	enrich	2216E	k	-				intertidal sediment	Harlesiel
Flavobacterium gelidilacus									
***F. gelidilacus* R-8899 (99.3%)**				**-**	**-**	**-**	**-**	**microbial mat**	**Lake Ace, East ANT**
MGE_SAT_510	enrich	2216E		-				intertidal sediment	Harlesiel

Continued on next page

Table 2.S3 (continued)

	Cultivation		Physiology					Sampling	
Name	Method	Medium	$Kana^R$	Flexirubin	Iridescence	Gliding	Lysis	Type	Site
***Flavobacterium* sp. I**									
***F. johnsoniae* DSM 2064 (96.8%)**				**-**	**-**	**gl**	**-**	**river epilithon**	**River Taff, UK**
MAR_2010_30	96 pin	HaHa		-				sediment porewater	Sylt, West beach
***Flavobacterium* sp. II**									
***F. tegetincola* A103 (95.5%)**				**-**	**-**	**-**	**-**	**cyanobacterial mat**	**Lake Ace, East ANT**
MGE_SAT_384	enrich	2216E		-				intertidal sediment	Harlesiel
***Nonlabens* sp. I**									
***N. xylanidelens* SW256 (98.5%)**				+	**-**	**-**	**-**	**coastal seawater**	**Hope Cove, UK**
Hel_I_56	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
***Nonlabens* sp. II**									
***N. dokdonensis* DSW-6 (95.5%)**				**-**	**-**	**-**	**-**	**surface seawater**	**Dokdo Island, KR**
Hel_I_38	96 pin	HaHa		-		gl		surface seawater	Helgoland, Kabeltonne
Cytophagia									
Cyclobacterium marinum									
***C. marinum* DSM 745 (98.3%)**									
SRO_15	plating	2216E		-				intertidal sediment	Sylt, West beach
TBL_72_124	plating	MAL		-				intertidal sediment	Janssand, UF
Cyclobacterium amurskyense									
***C. amurskyense* KMM 6143 (100%)**									
TBL_14	plating	CAA		-				intertidal sediment	Sylt, Hausstrand
***Cyclobacterium* sp. I**									
***C. amurskyense* KMM 6143 (98.0%)**									
MAR_2009_87	plating	NAG		-				intertidal sediment	Sylt, Königshafen
Reichenbachiella faecimaris									
***R. faecimaris* PCP11 (98.9%)**									
MAR_2010_115	96 pin	HaHa		-				sediment porewater	Sylt, West beach
Sphingobacteria									
Lewinella marina									
***L. marina* MKG-38 (98.8%)**									
SRO_346	plating	RAM		-				intertidal sediment	Janssand, MF
SRO_484	plating	GAL		-				intertidal sediment	Janssand, UF

Table 2.S4 Strains within the novel candidate genera of this study and the 16S rRNA sequence identity with the next relative type strain. The strains were incubated in the medium (SYL, HaHa) and physiological characteristics were observed (Kana^R, kanamycin resistence; gl, gliding motility; Lysis, agar lysis). All strains isolated on HaHa medium were initially identified as Flavobacteriaceae by PCR screen.

	Cultivation		Sampling		Gliding	Taxonomy	
Name	Method	Medium	Type	Site		Next related strain	Identity
candidate gen. nov. A							
MAR_2009_75	plating	SYL	20 μm phytoplankton	Sylt, List	gliding	*Pseudozobellia thermophila* KMM 3531	94.7%
						Costertonia aggregata KOPRI 13342	88.2%
candidate gen. nov. B							
Hel_48	96 pin	HaHa	surface seawater	Helgoland, Kabeltonne	gliding	*Aequorivita antarctica* SW49	90.1%
						Vitellibacter vladivostokensis KMM3516	90.5%
						Marixanthomonas ophiurae KMM 3046	90.4%
						Leeuwenhoekiella aequorea R7695	91.6%
candidate gen. nov. C							
MAR_2010_72	96 pin	HaHa	sediment porewater	Sylt, West beach		*Gelidibacter algens* ACAM 536	94.0%
MAR_2010_78	96 pin	HaHa	sediment porewater	Sylt, West beach		*Subsaximicrobium wynnwilliamsii* G#7	93.8%
MAR_2010_97	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_105	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_106	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_107	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_111	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_113	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_119	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_165	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_169	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_184	96 pin	HaHa	sediment porewater	Sylt, West beach			
MAR_2010_199	96 pin	HaHa	sediment porewater	Sylt, West beach			
candidate gen. nov. D							
MAR_2010_188	96 pin	HaHa	sediment porewater	Sylt, West beach		*Winogradskyella thalassocola* KMM 3907	90.5%
						Sediminibacter furfurosus MAOS-86	94.0%
						Algibacter lectus KMM 3902	90.0%
candidate gen. nov. E							
Hel_9	96 pin	HaHa	surface seawater	Helgoland, Kabeltonne	gliding	*Winogradskyella thalassocola* KMM 3907	91.1%
Hel_10	96 pin	HaHa	surface seawater	Helgoland, Kabeltonne	gliding	*Sediminibacter furfurosus* MAOS-86	91.4%
						Algibacter lectus KMM 3902	91.2%
candidate gen. nov. F							
MAR_2010_118	96 pin	HaHa	sediment porewater	Sylt, West beach		*Snuella lapsa* JC2132	93.2%
						Yeosuana aromativorans GW1-1	94.3%
						Meridianimaribacter flavus NH57N	94.7%
candidate gen. nov. G							
MAR_2010_10	96 pin	HaHa	sediment porewater	Sylt, West beach		*Snuella lapsa* JC2132	93.7%
						Yeosuana aromativorans GW1-1	92.7%
						Meridianimaribacter flavus NH57N	95.0%

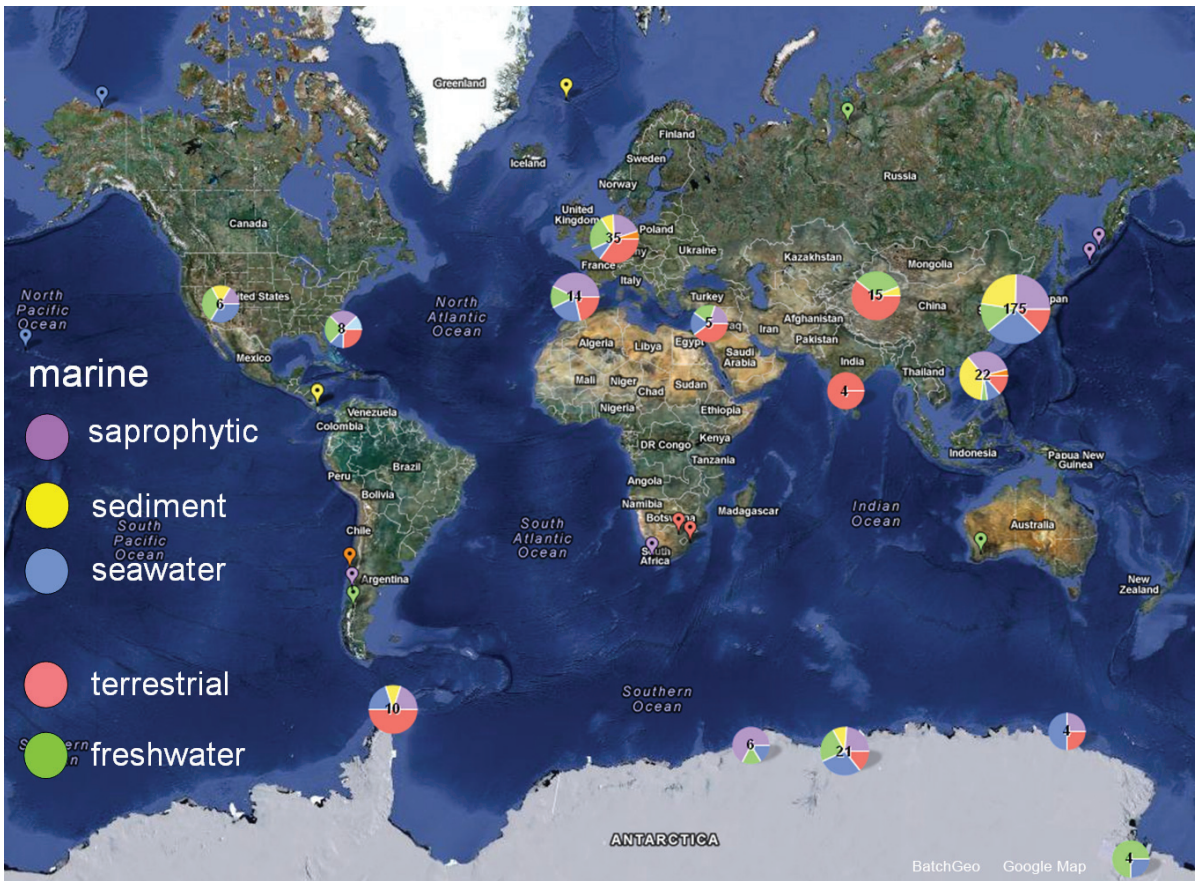

Figure 2.S1 Sampling sites of *Flavobacteriaceae* type strains. Environment of the sampling sites from which type strains were isolated as listed in the *List of Prokaryotic names with Standing in Nomenclature* (http://www.bacterio.cict.fr, 06.2012) (Euzéby, 1997). Numbers in pie charts represent the number of type strains.

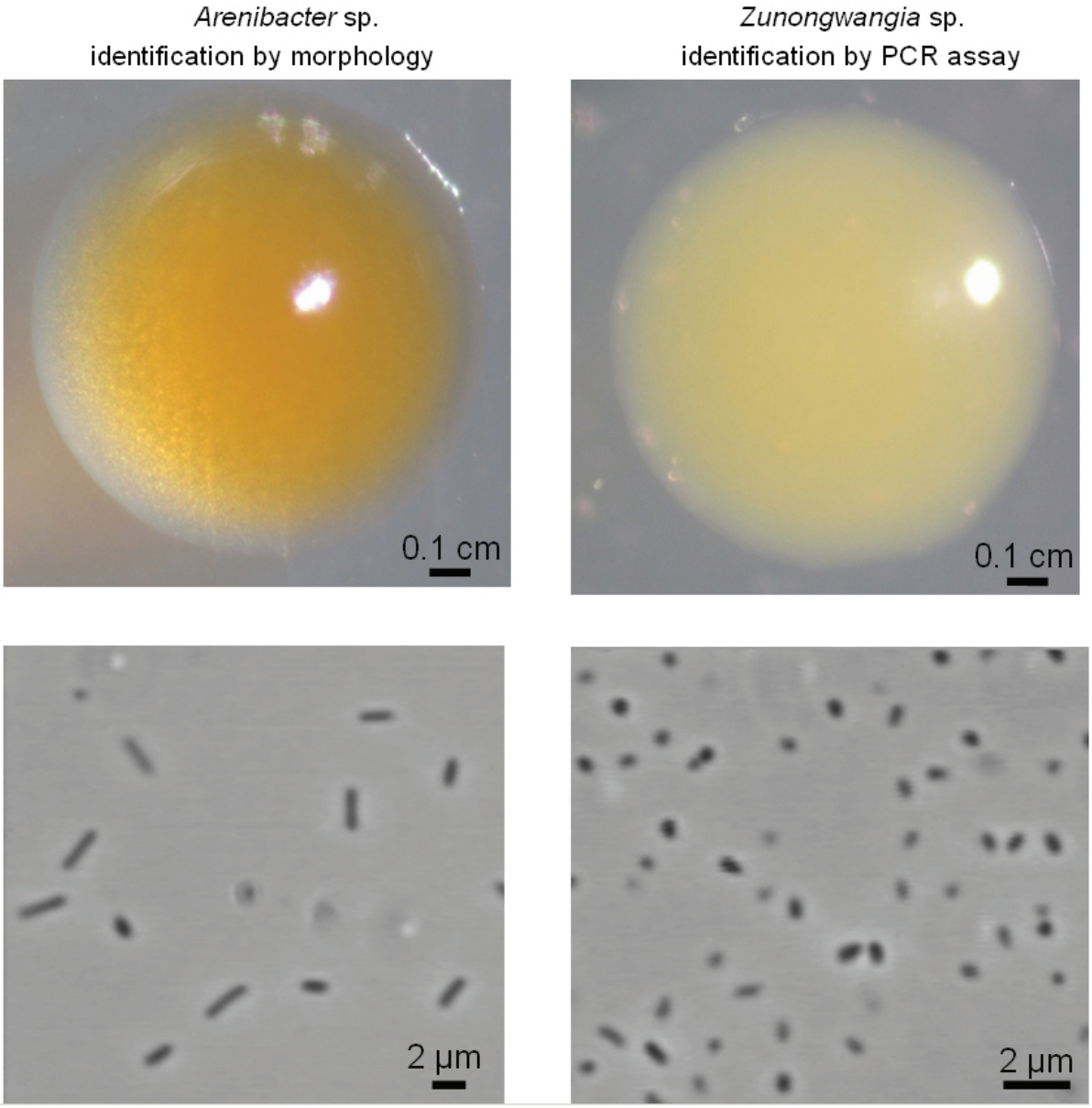

Figure 2.S2 Colonies on agar plates and cell morphology of *Arenibacter* sp. (upper and lower left) and *Zunongwangia* sp. (upper and lower right) at room temperature after seven days of incubation. *Arenibacter* spp. had prominent characteristics of cell- (long rod shaped) and colony morphology (yellow-orange color), in contrast to *Zunongwangia* spp.. *Arenibacter* spp. were often identified as *Flavobacteriaceae* by the traditional observation of colony color and rod-shaped cell morphology, whereas *Zunongwangia* spp. were identified as *Flavobacteriaceae* by the *Flavobacteria-Cytophagia* specific PCR assay only.

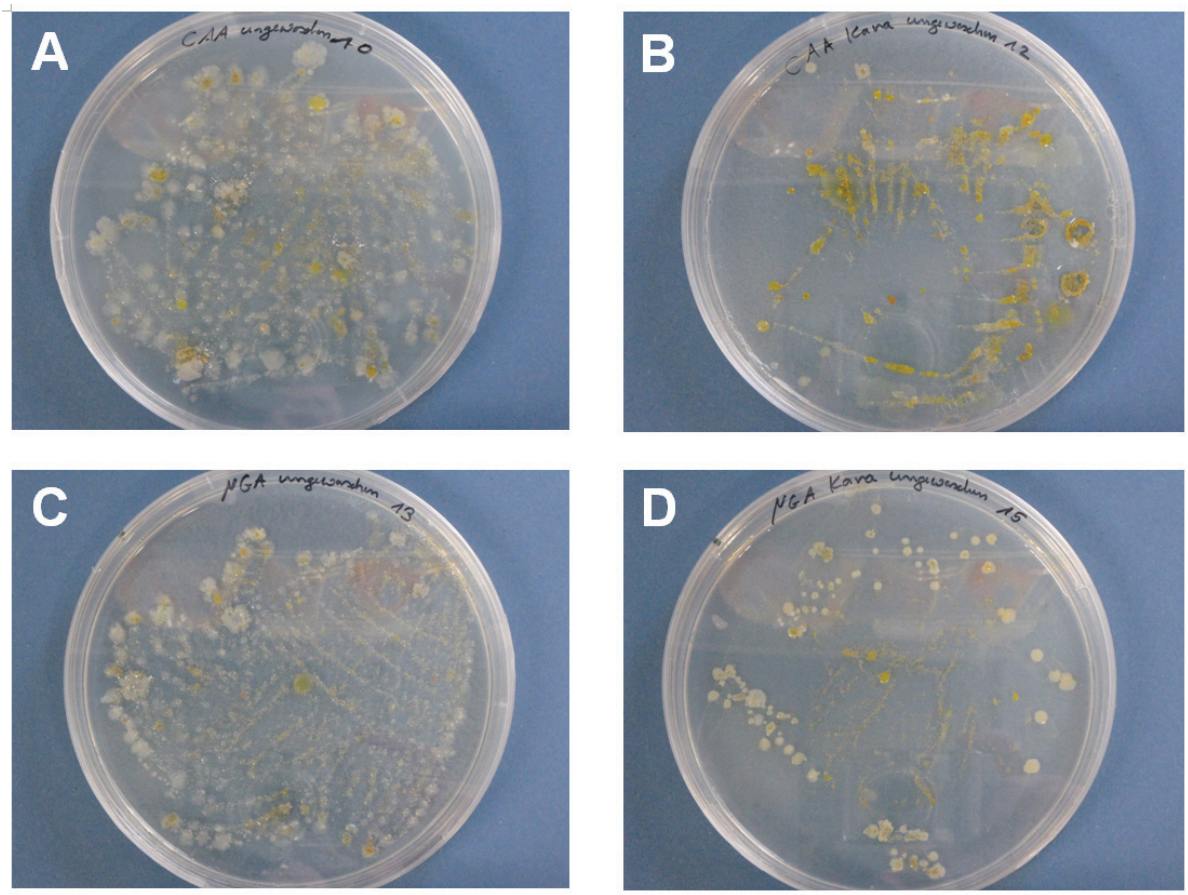

Figure 2.S3 Incubated sediment of Königshafen on agar, supplemented with casamino acids (CAA, A and B) or N-acetylglucosamine (NAG, C and D). The sediment was incubated without (A and C) or with kanamycin (Kana, B and D) to test for the selective effect of the antibiotic kanamycin.

Distribution of seawater on solid agar plates

An aliquot of 0.5 mL seawater sample was placed onto the agar in the middle of the Petri dish and even distributed with sterile glass beads. Seawater aliquots of less than 0.3 mL were diluted in sterile artificial seawater and aliquots of more than 0.3 mL were successively distributed on the agar.

Distribution of sediment on solid agar plates

Depending on the amount of porewater, the sediment sample was mixed with up to 0.3 mL sterile artificial seawater on the solid agar. The sediment was distributed on the solid agar using an inoculating loop with the following scheme (Fig. 2.S4). Particular attention was given to avoid scratching or plowing of the agar surface.

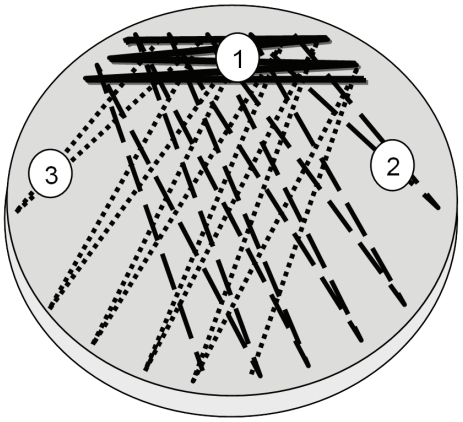

Figure 2.S4 Distribution of sediment on agar plates diluted by an inoculation loop following the scheme (1–3).

Bacteria attached to plant and animal specimens

Plants or pieces of plants were placed into a 50 mL polypropylene tube, chopped with a sterile scalpel and washed with sterile artificial seawater before distributing them on the solid agar. Animal specimens (e.g. small crab shells, seashells) were rinsed with seawater first, followed by sterile artificial seawater before placing them on the solid agar.

KOH test (test for bathochromic shift)

To test for a bathochromic shift, a colony was deposited on a slide and covered with a drop of KOH. In a positive test, the color of the biomass changed from yellow to red or orange to red-brown upon KOH addition and reverted back upon acidification with two drops of 10% (v/v) HCl (Fig. 2.S5).

Figure 2.S5 Bathochromic shift of flexirubin type pigments caused by KOH treatment, observable by a shift in colony color from yellow to orange of the strain *Aquimarina* sp. TBL_9.

96 pin replicator

Using a 96 pin replicator enables a transfer of 1 μL per pin (Winkelmann and Harder, 2009) on 96 defined positions on a 14 cm Petri dish with solid agar (Fig. 2.S6). A sterile 14 cm Petri dish was filled with 50 mL of seawater or porewater sample. The volume of 50 mL was necessary to fill the bottom of the large Petri dish completely. For dilution series, 5 mL of the diluted water sample were mixed with 45 mL artificial seawater in a fresh, sterile Petri dish. Bacteria of the water sample were transferred onto the agar plate by dipping the sterile 96 pin replicator into the water sample without touching the bottom of the Petri dish, followed by gently touching the surface of the solid agar.

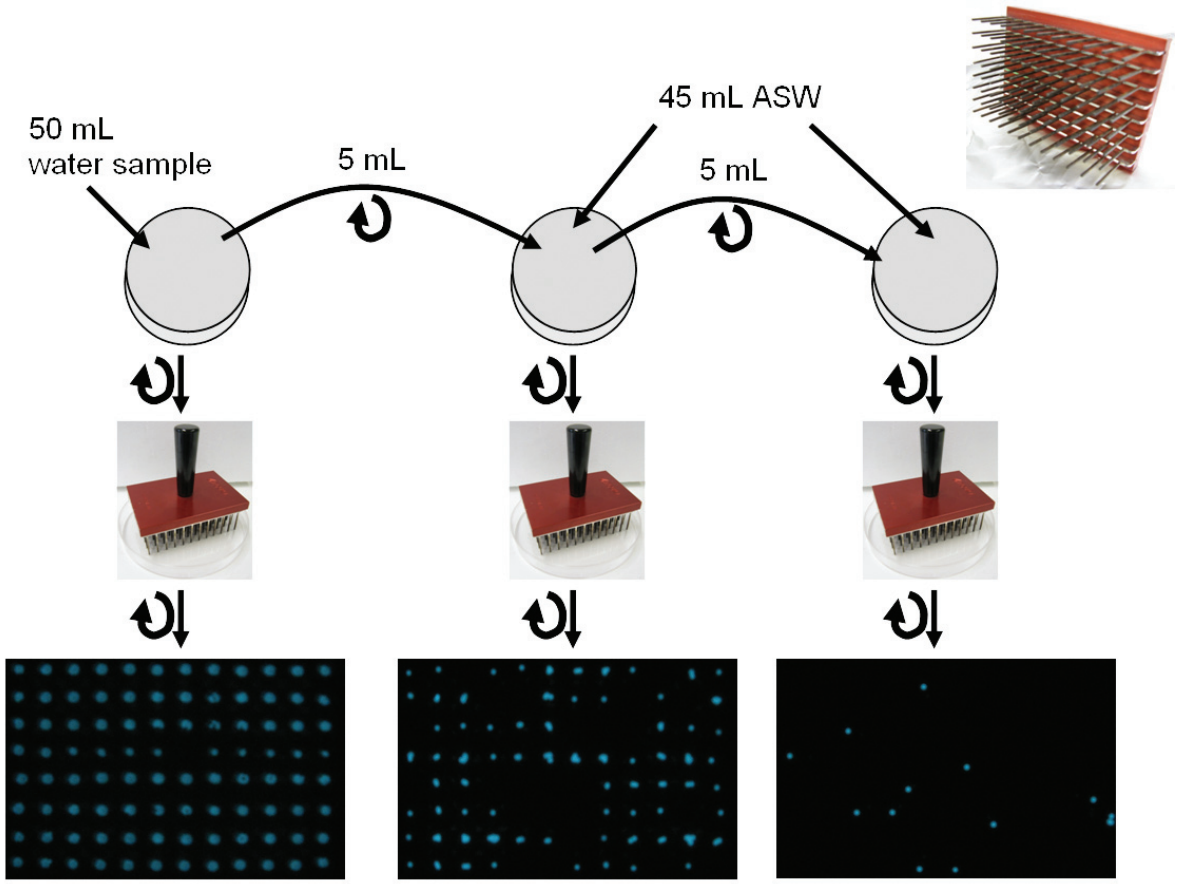

Figure 2.S6 Distribution of seawater or porewater at defined positions on solid agar medium using the 96 pin replicator, modified after Winkelmann and Harder (2009). The water sample was diluted with sterile artificial seawater (ASW) before transferring on agar plates. Blue dots represent luminescent colony forming units of *Photobacterium* sp. as control for a random distribution of cells with the 96 pin replicator. The spinning arrow indicates the gentle mixing of water samples in the Petri dish by horizontal shaking.

HaHa medium (agar plates)

Artificial seawater medium:
after (Widdel and Bak, 1992) modified by (Winkelmann and Harder, 2009)

1. Prepare 2x ASW by dissolving the basal salts in 1L ultra pure water

	1 L 1x ASW	1 L 2x ASW
NaCl	26.37 g	52.74 g
$NaHCO_3$	0.19 g	0.038 g
$CaCl_2 \cdot 2\,H_2O$	1.47 g	2.94 g
KCl	0.72 g	1.44 g
KBr	0.10 g	0.20 g
H_3BO_3	0.02 g	0.04 g
$SrCl_2$	0.02 g	0.04 g
NaF	0.003 g	0.006 g

2. Wash 12.6 g Bacto™ agar (18 g/L)
- a) add bacto agar in a 1 L bottle
- b) add 600 mL ultra pure water
- c) clean inner surface of the bottle with ultra pure water (final volume $<$ 800 mL)
- d) let agar settle
- e) remove the overlaying water
- repeat twice from b)

! The final volume of the washed agar should be less than 300 mL !

3. Add 350 mL of 2x ASW

4. ! Add magnetic stir bar !

5. Add HEPES (50 mM, pH 7.5) 9.92 g

6a. Fill up to 650 mL with MilleQ
(6b. Check $6.5 < \text{pH} < 7.0$)

7a. Direct before autoclavation, mix agar and liquid
7b. Autoclave, cool (60 ℃, keep at 55 ℃ in a pre-heated water bath)

8. Add sterile from the following stock solutions:

1.4 mL	Trace-element-solution	(autoclaved)
0.7 mL	Se-W-solution	(sterile filtered in PP tubes)
7.0 mL	KH_2PO_4-solution	(50 g/L, autoclaved)
3.5 mL	NH_4Cl-solution	(50 g/L, autoclaved)

9. Add carbon sources from the following stock solutions

3.5 mL	Glucose	(100 g/L, sterile filtered)
3.5 mL	Cellobioses	(100 g/L, sterile filtered)
3.5 mL	Yeast Extract (BioChemica)	(100 g/L, sterile filtered)
3.5 mL	Casaminoacids (Difco)	(100 g/L, sterile filtered)
3.5 mL	Tryptone Pepton (Difco)	(100 g/L, sterile filtered)

10. Adjust pH to pH 7.5 with 1 M HCl or 1 M NaOH (autoclaved)

11. Add from the following stock solutions

7.9 mL	$MgCl_2 \cdot 6\,H_2O$	(500 g/L, autoclaved, 5.67 g/L)
9.5 mL	$MgSO_4 \cdot 7\,H_2O$	(500 g/L, autoclaved, 6.8 g/L)

12. Add MilleQ water to a final volume of 700 mL, avoid bubbles (autoclaved)

Trace-element-solution (0.5 L)

Start with 400 mL water, add	
Na_2-EDTA	2600 mg
$FeSO_4 \cdot 7\,H_2O$	1050 mg
dissolve and add	
H_3BO_3	15 mg
$MnCl_2 \cdot 4\,H_2O$	50 mg
$CoCl_2 \cdot 6\,H_2O$	95 mg
$NiCl_2 \cdot 6\,H_2O$	12 mg
$CuCl_2 \cdot 2\,H_2O$	5 mg
$ZnSO_4 \cdot 7\,H_2O$	72 mg
$Na_2MoO_4 \cdot 2\,H_2O$	18 mg
Adjust pH to 6.0 with 5 M NaOH	
Autoclave	

Se-W-solution

Dissolve in 0.5 L water	
NaOH	1000 mg
$Na_2SeO_3 \cdot 5\,H_2O$	9 mg
$Na_2WoO_4 \cdot 2\,H_2O$	9 mg
sterile filter in PP tubes	
Autoclave fresh	

References

Abell, G. C. J. and Bowman, J. P. (2005). Colonization and community dynamics of class *Flavobacteria* on diatom detritus in experimental mesocosms based on Southern Ocean seawater. *FEMS Microbiol Ecol* **53**, 379–391.

Alongi, D. (1998). *Coastal ecosystem processes*. CRC Press, Boca Raton, USA.

Alonso, C., Warnecke, F., Amann, R. and Pernthaler, J. (2007). High local and global diversity of *Flavobacteria* in marine plankton. *Environ Microbiol* **9**, 1253–1266.

Barbeyron, T., Carpentier, F., L'Haridon, S., Schüler, M., Michel, G. and Amann, R. (2008). Description of *Maribacter forsetii* sp. nov., a marine *Flavobacteriaceae* isolated from North Sea water, and emended description of the genus *Maribacter*. *Int J Syst Evol Microbiol* **58**, 790–797.

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H., Knaust, F. et al. (2006). Whole genome analysis of the marine bacteroidetes '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**, 2201–2213.

Bernardet, J.-F. (2010). *Bergey's Manual of Systematic Bacteriology. The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes Vol 4*, chapter Class II. *Flavobacteriia* class. nov. Krieg, N.R., Staley, J.T.,

Brown, D.R., Hedlund, B.P., Paster, B.J., Ward, N.L. et al. (eds), pp. 106–314. Springer, New York.

Brandt, P., Gerdts, G., Boersma, M., Wiltshire, K. H. and Wichels, A. (2010). Comparison of different DNA-extraction techniques to investigate the bacterial community of marine copepods. *Helgoland Mar Res* **64**, 331–342.

Bruns, A., Rohde, M. and Berthe-Corti, L. (2001). *Muricauda ruestringensis* gen. nov., sp. nov., a facultatively anaerobic, appendaged bacterium from German North Sea intertidal sediment. *Int J Syst Evol Microbiol* **51**, 1997–2006.

Cantarel, B., Coutinho, P., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009). The Carbohydrate-Active enZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* **37**, D233–D238.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M. et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37**, D141–D145.

Doucet, S. M. and Meadows, M. G. (2009). Iridescence: a functional perspective. *J R Soc Interface* **6**, S115–S132.

Eilers, H., Pernthaler, J., Glöckner, F. O. and Amann, R. (2000). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66**, 3044–3051.

Euzéby, J. P. (1997). List of bacterial names with standing in nomenclature: A folder available on the Internet. *Int J Syst Bacteriol* **47**, 590–592.

Fautz, E. and Reichenbach, H. (1979). Biosynthesis of flexirubin: incorporation of precursors by the bacterium *Flexibacter elegans*. *Phytochemistry* **18**, 957–959.

Fautz, E. and Reichenbach, H. (1980). A simple test for flexirubin-type pigments. *FEMS Microbiol Lett* **8**, 87–91.

Flint, K. P. (1985). A note on a selective agar medium for the enumeration of *Flavobacterium* species in water. *J Appl Bacteriol* **59**, 561–566.

Frette, L., Jørgensen, N. O. G., Irming, H. and Kroer, N. (2004). *Tenacibaculum skagerrakense* sp. nov., a marine bacterium isolated from the pelagic zone in Skagerrak, Denmark. *Int J Syst Evol Microbiol* **54**, 519–524.

Gao, H., Matyka, M., Liu, B., Khalili, A., Kostka, J. E., Collins, G., Jansen, S., Holtappels, M., Jensen, M. M., Badewien, T. H. et al. (2012). Intensive and extensive nitrogen loss from intertidal permeable sediments of the Wadden Sea. *Limnol Oceanogr* **57**, 185–198.

Gómez-Pereira, P. R., Fuchs, B. M., Alonso, C., Oliver, M. J., van Beusekom, J. E. E. and Amann, R. (2010). Distinct flavobacterial communities in contrasting water masses of the North Atlantic Ocean. *ISME J* **4**, 472–487.

Grossart, H. P., Schlingloff, A., Bernhard, M., Simon, M. and Brinkhoff, T. (2004). Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. *FEMS Microbiol Ecol* **47**, 387–396.

Hansen, G. H., Bergh, O., Michaelsen, J. and Knappskog, D. (1992). *Flexibacter ovolyticus* sp. nov., a pathogen of eggs and larvae of

atlantic halibut, *Hippoglossus hippoglossus* L. *Int J Syst Bacteriol* **42**, 451–458.

Janssen, P. H., Yates, P. S., Grinton, B. E., Taylor, P. M. and Sait, M. (2002). Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. *Appl Environ Microbiol* **68**, 2391–2396.

Johansen, J., Nielsen, P. and Sjohølm, C. (1999). Description of *Cellulophaga baltica* gen. nov., sp. nov., and *Cellulophaga fucicola* gen. nov., sp. nov. and reclassification of [*Cytophaga*] *lytica* to *Cellulophaga lytica* gen. nov., comb. nov. *Int J Syst Bacteriol* **49**, 1231–1240.

Jukes, T. H. and Cantor, C. R. (1969). *Mammalian protein metabolism. Munro, H.N. (ed)*, chapter Evolution of protein molecules. Academic Press, New York.

Kientz, B., Vukusic, P., Luke, S. and Rosenfeld, E. (2012). Iridescence of a marine bacterium and classification of prokaryotic structural colors. *Appl Environ Microbiol* **78**, 2092–2099.

Kirchman, D. L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41**, e1.

Klippel, B., Lochner, A., Bruce, D. C., Davenport, K. W., Detter, C., Goodwin, L. A., Han, J., Han, S. S., Hauser, L., Land, M. L. et al. (2011). Complete genome sequences of *Krokinobacter* sp. strain

4H-3-7-5 and *Lacinutrix* sp. strain 5H-3-7-4, polysaccharide-degrading members of the family *Flavobacteriaceae*. *J Bacteriol* **193**, 4545–4546.

Llobet-Brossa, E., Rosselló-Móra, R. and Amann, R. (1998). Microbial community composition of Wadden Sea sediments as revealed by fluorescence *in situ* hybridization. *Appl Environ Microbiol* **64**, 2691–2696.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G. et al. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Meadows, M. G., Butler, M. W., Morehouse, N. I., Taylor, L. A., Toomey, M. B., McGraw, K. J. and Rutowski, R. L. (2009). Iridescence: views from many angles. *J R Soc Interface* **6**, S107–S113.

Musat, N., Werner, U., Knittel, K., Kolb, S., Dodenhof, T., van Beusekom, J. E. E., de Beer, D., Dubilier, N. and Amann, R. (2006). Microbial community structure of sandy intertidal sediments in the North Sea, Sylt-Rømø Basin, Wadden Sea. *Syst Appl Microbiol* **29**, 333–348.

Muyzer, G., Teske, A., Wirsen, C. and Jannasch, H. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**, 165–172.

Nedashkovskaya, O. I., Vancanneyt, M., Dawyndt, P., Engelbeen, K., Vandemeulebroecke, K., Cleenwerck, I., Hoste, B., Mergaert, J., Tan, T.-L., Frolova, G. M. et al. (2005). Reclassification of [*Cytophaga*] *marinoflava* Reichenbach 1989 as *Leeuwenhoekiella marinoflava* gen. nov., comb. nov. and description of *Leeuwenhoekiella aequorea* sp. nov.. *Int J Syst Evol Microbiol* **55**, 1033–1038.

O'Sullivan, L. A., Rinna, J., Humphreys, G., Weightman, A. J. and Fry, J. C. (2006). Culturable phylogenetic diversity of the phylum '*Bacteroidetes*' from river epilithon and coastal water and description of novel members of the family *Flavobacteriaceae*: *Epilithonimonas tenax* gen. nov., sp. nov. and *Persicivirga xylanidelens* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **56**, 169–180.

Pfennig, N., and Trüper, H. G. (1981). *The Prokaryotes*, chapter Isolation of members of the families *Chromatiaceae* and *Chlorobiaceae*. Starr, M.P. and Stolp, H. and Trüper, H. G. and Balows, A. and Schlegel, H. G. (eds), pp. 279–289. Springer, Berlin, Germany.

Port, A., Gurgel, K.-W., Staneva, J., Schulz-Stellenfleth, J. and Stanev, E. V. (2011). Tidal and wind-driven surface currents in the German Bight: HFR observations versus model simulations. *Ocean Dynam* **61**, 1567–1585.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W. G., Peplies, J. and Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**, 7188–7196.

Riedel, T., Tomasch, J., Buchholz, I., Jacobs, J., Kollenberg, M., Gerdts, G., Wichels, A., Brinkhoff, T., Cypionka, H. and Wagner-Döbler, I. (2010). Constitutive expression of the proteorhodopsin gene by a flavobacterium strain representative of the proteorhodopsin-producing microbial community in the North Sea. *Appl Environ Microbiol* **76**, 3187–3197.

Rink, B., Martens, T., Fischer, D., Lemke, A., Grossart, H. P., Simon, M. and Brinkhoff, T. (2008). Short-term dynamics of bacterial

communities in a tidally affected coastal ecosystem. *FEMS Microbiol Ecol* **66**, 306–319.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Salaün, S., Kervarec, N., Potin, P., Haras, D., Piotto, M. and La Barre, S. (2010). Whole-cell spectroscopy is a convenient tool to assist molecular identification of cultivatable marine bacteria and to investigate their adaptive metabolism. *Talanta* **80**, 1758–1770.

Sapp, M., Wichels, A. and Gerdts, G. (2007). Impacts of cultivation of marine diatoms on the associated bacterial community. *Appl Environ Microbiol* **73**, 3117–3120.

Stackebrandt, E. and Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today* **33**, 152–155.

Stevens, H., Simon, M. and Brinkhoff, T. (2009). Cultivable bacteria from bulk water, aggregates, and surface sediments of a tidal flat ecosystem. *Ocean Dynam* **59**, 291–304.

Stevens, H., Stübner, M., Simon, M. and Brinkhoff, T. (2005). Phylogeny of *Proteobacteria* and *Bacteroidetes* from oxic habitats of a tidal flat ecosystem. *FEMS Microbiol Ecol* **54**, 351–365.

Suzuki, M., Nakagawa, Y., Harayama, S., and Yamamoto, S. (2001). Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bacteria: proposal for *Tenacibaculum* gen. nov. with *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amylolyticum* sp. nov. *Int J Syst Evol Microbiol* **51**, 1639–1652.

Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J. et al. (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336**, 608–611.

Teske, A., Brinkhoff, T., Muyzer, G., Moser, D. P., Rethmeier, J. and Jannasch, H. W. (2000). Diversity of thiosulfate-oxidizing bacteria from marine sediments and hydrothermal vents. *Appl Environ Microbiol* **66**, 3125–3133.

Vukusic, P. and Sambles, J. R. (2003). Photonic structures in biology. *Nature* **424**, 852–855.

Wichels, A., Würtz, S., Döpke, H., Schütt, C. and Gerdts, G. (2006). Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). *FEMS Microbiol Ecol* **56**, 102–118.

Widdel, F. and Bak, F. (1992). *The Prokaryotes Vol 2*, chapter Gram-negative mesophilic sulfate-reducing bacteria. Balows A., Trüper H.G., Dworkin M. and Harder W. (eds), pp. 3352–3378. Springer, Berlin, Germany.

Winkelmann, N. and Harder, J. (2009). An improved isolation method for attached-living *Planctomycetes* of the genus *Rhodopirellula. J Microbiol Meth* **77**, 276–284.

Yarza, P., Ludwig, W., Euzéby, J., Amann, R., Schleifer, K.-H., Glöckner, F. O. and Rosselló-Móra, R. (2010). Update of the All-Species Living Tree Project based on 16S and 23S rRNA sequence analyses. *Syst Appl Microbiol* **33**, 291–299.

Zinger, L., Amaral-Zettler, L. A., Fuhrman, J. A., Horner-Devine, M. C., Huse, S. M., Welch, D. B. M., Martiny, J. H., Sogin, M., Boetius, A. and Ramette, A. (2011). Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. *PLoS ONE* **6**, e24570.

ZoBell, C. E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J Mar Res* **4**, 42–75.

ZoBell, C. E. (1946). *Marine microbiology. A monograph of hydrobacteriology.* Chronica Botanica Co., Waltham, Mass. U.S.A.

Zubkov, M. V., Fuchs, B. M., Archer, S. D., Kiene, R. P., Amann, R. and Burkill, P. H. (2001). Linking the composition of bacterioplankton to rapid turnover of dissolved dimethylsulphoniopropionate in an algal bloom in the North Sea. *Environ Microbiol* **3**, 304–311.

Polaribacter strain cultivated in a polystyrene tube on marine HaHa_100 medium.

Chapter 3

Dilution cultivation of marine heterotrophic bacteria benefiting from a coastal diatom bloom

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R.L.H., B.M.F., H.T., R.A. and J.H. designed research and project outline. R.L.H. and J.H. developed the new medium and cultivation procedure. R.L.H. and B.M.F. performed flow cytometry. C.M.B. and B.M.F. performed CARD-FISH. C.M.B. and R.L.H. performed FISH probe testing on isolates. R.L.H. performed isolation, 16S rRNA and proteorhodopsin amplification, and gDNA extraction. B.M.F., H.T. and R.A. organized genome sequencing. A.J.M. and H.T. performed genomic and metagenomic analysis. R.L.H., H.T., R.A. and J.H. performed phylogenetic analysis. R.L.H., C.M.B., B.M.F., A.J.M., H.T., R.A. and J.H. conceived, wrote and edited the manuscript.

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3.1 Abstract

Planktonic bacteria respond notably to phytoplankton primary production, but their role in the remineralization of algal biomass is poorly understood. During a spring phytoplankton bloom in the German Bight of the North Sea in 2009, we observed high abundances of yet uncultivated representatives of the genera *Formosa*, *Polaribacter* (*Flavobacteria*), and *Reinekea* (*Gammaproteobacteria*). In order to obtain isolates, we sampled at the same location and time of the year during the 2010 spring phytoplankton bloom. Using a newly devised artificial seawater medium with environmental-like nutrient concentrations we could attain a culturability of 35% of the bacterioplankton. Twenty-five novel isolates were gained, belonging to *Flavobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria*, including *Formosa*, *Polaribacter*, and *Reinekea* isolates. The 16S rRNA gene sequences of these isolates exhibited identities of up to 99.8% when compared to full-length 16S rRNA gene clones of bacterioplankton of the 2009 bloom. Likewise, draft genomes of selected isolates could recruit reads of metagenomes from bacterioplankton of the 2009 spring bloom that had $\geqslant$ 95% nucleotide identity which covered the draft genomes by 94% (*Formosa* sp.), 90% (*Reinekea* sp.), and 50% (*Polaribacter* sp.). Based on these data we argue that the isolates retrieved in this study are representatives of ecologically relevant clades catalyzing the remineralization of coastal diatom-dominated phytoplankton biomass.

3.2 Introduction

The response of heterotrophic bacterioplankton to algal blooms is dynamic (Azam, 1998; Sapp et al., 2007b). In a recent study it was shown how a diatom-dominated spring phytoplankton bloom in the German Bight of the North Sea in 2009 changed the bacterioplankton community composition by exerting a positive selection for bacteria with the capacity to decompose phytoplankton biomass. These bacteria constituted distinct clades that were characterized by notably different substrate spectra, in particular with respect to polysaccharide degradation (Teeling et al., 2012). Polysaccharides are major constituents of blooming marine microalgae such as diatoms and haptophytes. When such algae disintegrate, these polysaccharides are released and become available as substrates to the bacterioplankton community. Some of these substrates are easier to degrade than others and thus are preferentially degraded by specialized bacteria, which can result in a succession of distinct blooming bacterioplankton clades. During the 2009 spring algae bloom in the German Bight, a swift succession of *Ulvibacter*, *Formosa*, and *Polaribacter* (*Flavobacteria*), *Reinekea* and SAR92 (*Gammaproteobacteria*) was observed, in which each of these clades reached 15% to 25% of the total picoplankton community (Teeling et al., 2012).

It is known that marine *Flavobacteria* play a pivotal role in the decomposition of complex organic matter (Kirchman, 2002), in particular of proteins and polysaccharides (Bauer et al., 2006; Martens et al., 2011; Gómez-Pereira et al., 2012; Teeling et al., 2012; Fernández-Gómez et al., 2013). *Flavobacteria* use TonB-dependent transporters for the uptake of algae-derived oligosaccharides (Bauer et al., 2006; Schauer et al., 2008; Gómez-Pereira et al., 2012; Teeling et al., 2012), whereas *Gammaproteobacteria* such as *Reinekea* and *Alphaproteobacteria* mainly use TRAP and ABC transporters for the uptake of monomeric carbohydrates and amino acids

that become available during the initial decomposition of algal biomass (Mulligan et al., 2011; Schneider et al., 2012; Teeling et al., 2012). Our comprehension of such microbial niches in nature is limited by the existing knowledge of the underlying biochemistry (Hugenholtz and Tyson, 2008), which is reflected in the high proportions of genes without known functions in environmental microbes (Venter et al., 2004; Yooseph et al., 2007). Consequently, targeted studies on cultivated strains are a necessity for obtaining a more complete picture of the functional repertoires and activities of microbes that have been identified in cultivation-independent studies (reviewed by Glöckner and Joint, 2010; Joint et al., 2010; Overmann, 2010). However, since most *in situ* surveys of microbial communities lack accompanying isolates, it is one of the principal challenges of environmental microbiologists to develop strategies that allow cultivation of ecologically relevant microorganisms (reviewed by Schloss and Handelsman, 2004; Glöckner and Joint, 2010; Joint et al., 2010).

Numerous marine bacteria from many phyla have already been brought into culture. This work was pioneered by Bernhard Fischer (1894) and Claude Ephraim ZoBell (1946). Using ZoBell's marine agar, Pinhassi et al. (1997) could obtain phytoplankton-associated *Flavobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria*. Furthermore, these authores showed their dominance and seasonality using whole-genome DNA hybridization. A synthetic seawater agar, supplemented with inorganic nitrogen and phosphorus compounds in micromolar concentrations allowed Eilers et al. to cultivate representatives of the cosmopolitan NOR5 clade of *Gammaproteobacteria* (Eilers et al., 2001). Button's technique and theory of dilution cultivation in sterilized oligotrophic seawater (Button et al., 1993) ultimately opened the field of high-throughput cultivation and allowed to obtain novel *Proteobacteria* species that until then belonged to the uncultured part of the marine bacterioplankton (Connon and Giovannoni, 2002). An improvement

of their medium by supplementing the sterilized seawater with inorganic nitrogen and phosphorus compounds and a defined mixture of organic carbon compounds in micromolar concentrations, allowed the successful cultivation of '*Candidatus* Pelagibacter ubique' (Rappé et al., 2002) – a decade after the discovery of the highly abundant SAR11 (Giovannoni et al., 1990).

The goal of this study was to obtain isolates representative of bacterioplankton clades highly abundant during a diatom-dominated phytoplankton bloom in 2009 near the North Sea island Helgoland in the German Bight (Teeling et al., 2012). For that we sampled a similar spring phytoplankton bloom at the same location in 2010. Since many bacterial species cannot be cultivated on agar plates (Staley and Konopka, 1985; Pedrós-Alió, 2006), an artificial seawater (ASW) medium was designed mimicking *in situ* carbon, nitrogen, and phosphorus concentrations. The medium composition was derived from the ASW medium of Hahnke and Harder, which enabled the aerobic cultivation of *Proteobacteria*, *Flavobacteria* and *Actinobacteria*, without showing a distorting cultivation-induced *Gammaproteobacteria* shift (Hahnke and Harder, 2013). As a result, we obtained an overall culturability of 35% of the total bacterioplankton, and could isolate representatives of the genera *Formosa*, *Reinekea* and *Polaribacter*. Using sequence-based comparisons of 16S rRNA genes, proteorhodopsins and draft genomes with 16S rRNA gene clone libraries and metagenomes of bacterioplankton from the 2009 spring phytoplankton bloom, we could furthermore demonstrate the relevance of these isolates as key players during coastal diatom-dominated phytoplankton blooms in the North Sea.

3.3 Material and methods

Medium preparation

Artificial seawater (ASW) and all media were prepared with sterile filtered (0.2 μm polycarbonate filter) and autoclaved ultra pure water (Aquintus system, membraPure, Berlin, Germany) with a resistivity of 18.2 Ω cm, a total organic carbon of less than 5 ppb, pyrogens of less than 0.001 EU/mL and heavy metals of less than 0.1 ppb.

The ASW agar HaHa was prepared as described previously (Hahnke and Harder, 2013). The ASW medium was prepared in a Widdel flask (Widdel and Bak, 1992) modified for an aerobic medium of large volume, with low substrate concentrations and without volatile components (suppl. Fig. 3.S1). ASW was prepared following the recipe of Widdel and Bak (1992) as described by Winkelmann and Harder (2009) (see suppl. on page 160). Basal salts: 26.37 g NaCl, 5.67 g $MgCl_2 \cdot 6\,H_2O$, 6.8 g $MgSO_4 \cdot 7\,H_2O$, 1.47 g $CaCl_2 \cdot 2\,H_2O$, 0.72 g KCl, 0.10 g KBr, 0.02 g H_3BO_3, 0.02 g $SrCl_2$, 0.003 g NaF, were dissolved in 1 L autoclaved ultra pure water. After autoclaving in the modified Widdel flask, the ASW was tempered at room temperature and supplemented with 2 mL trace element solution (containing per liter ultra pure water: $FeSO_4 \cdot 7\,H_2O$, 2.1 g; Na_2−EDTA, 5.2 g; H_3BO_3, 30 mg; $MnCl_2 \cdot 4\,H_2O$, 100 mg; $CoCl_2 \cdot 6\,H_2O$, 190 mg; $NiCl_2 \cdot 6\,H_2O$, 24 mg; $CuCl_2 \cdot 2\,H_2O$, 10 mg; $ZnSO_4 \cdot 7\,H_2O$, 144 mg; $Na_2MoO_4 \cdot 2\,H_2O$, 36 mg; pH adjusted to 6.0 with 5 M NaOH (Pfennig et al., 1981) and 0.7 mL SeW solution (Widdel and Bak, 1992). The HaHa medium was supplemented with the sterile filtered (0.2 μm filter, Minisart, Sartorius, Göttingen, Germany) carbon sources glucose, cellobiose, yeast extract, peptone and casamino acids at 0.6 mg/L each (Hahnke and Harder, 2013), 1 mL NH_4Cl (0.2 g/L, autoclaved), 0.7 mL KH_2PO_4 (0.02 g/L, autoclaved) providing 100 μM carbon, 3.3 μM ammonium and 0.16 μM phosphate. The HaHa_100 medium

was supplemented with the same carbon sources, but at a concentration of 0.1 g/L each, 4 mL NH_4Cl (0.2 g/L, autoclaved), 10 mL KH_2PO_4 (2 g/L, autoclaved) providing a final concentration of 16.8 mM carbon, 15 μM ammonium and 16 μM phosphate. Flow injection analysis (Hall and Aller, 1992) revealed an ammonium concentration of 106 $\pm$ 0.7 μM, due to the addition of yeast extract (210 $\pm$ 7 μmol/0.5 g), peptone (120 $\pm$ 7 μmol/0.5 g), and casamino acids (185 $\pm$ 5 μmol/0.5 g) (suppl. Tab. S3.S2) . The HaHa_100V medium was identical to the HaHa_100 medium with the addition of sterile filtered (0.2 μm filter, Minisart, Sartorius) 1 mL 7-vitamin solution (Winkelmann and Harder, 2009), 1 mL vitamin B_{12} solution (Widdel and Bak, 1992), 1 mL thiamine solution (Winkelmann and Harder, 2009), and 1 mL riboflavin solution (Winkelmann and Harder, 2009). The medium was buffered with 2 mM $NaHCO_3$ (Widdel and Bak, 1992) at pH 7.5. Evaporated water was replaced with autoclaved ultra pure water. The ASW had a salinity of 34‰ S, comparable to the euhaline (> 30‰ S) sampling site (Radach et al., 1990).

Sampling

Untreated surface seawater was sampled from the station 'Kabeltonne' near the North Sea island Helgoland in the German Bight (54° 10' 58.3" N, 7° 53' 19.9" E, Helgoland Roads) at high tide on 20 April 2010 (T = 6.4 ℃, pH 7.8) and on 2 September 2010 (T = 15.4 ℃, pH 7.9). Seawater samples were transported in sterile 1 L Schott glass bottles to the laboratory at *in situ* temperature and processed within 30 minutes.

Determination of the microbial community

The total microbial cell counts were determined by DAPI (4',6-diamidino-2-phenylindole) staining, and specific microbial populations were determined by catalyzed reporter deposition-fluorescence *in situ* hybridization

(CARD-FISH) as described previously (Teeling et al., 2012). Probes including competitor and helpers are listed in supplementary (suppl. Tab. 3.S1).

Dilution cultivation and incubation

The seawater sample was diluted to near extinction in ASW, directly after sampling (suppl. Fig. 3.S2). Since the amount of cultivable microorganisms was uncertain, 100 μL of seawater (aliquots of 1 nL to 100 nL) were distributed with 1 mL syringes (styrene-free, DEHP-, latex- und silicone-oil-free, tuberculin, NORM-JECT, HSW, Germany) and sterile 0.90 × 70 mm needles (DEHP-, latex- and PVC-free, Sterican, B Braun, Germany) into 17 mL polystyrene tubes (Greiner Bio-One, Austria) with 10 mL ASW medium (HaHa medium, 100 μM carbon) to an average inoculum of 0.5 to 50 cells per tube. The polystyrene tubes were robust for a later transport, allowed diffusion of oxygen for aerobic cultivation, and a visual inspection of optical changes. The dilution cultures and agar plates were incubated in the dark at 12 ℃ (April seawater) or 22 ℃ (September seawater) for three months. During the incubation the salinity of 34‰ S and the pH of 7.5 of the ASW medium remained unchanged and precipitates were not observed.

Detection of growth

Cell densities were determined by flow cytometry or fluorescence microscopy. One milliliter of each enrichment was fixed with 37% formaldehyde (v/v) to a final concentration of 1% (v/v) for one hour at room temperature. Samples were diluted with autoclaved and filtered (0.2 μm filter, Minisart, Sartorius, Göttingen, Germany) ASW and stained with 1× SYBRGreen (Applied Biosystems, Darmstadt, Germany) and processed by flow cytometry (FACSCalibur, Becton Dickenson, BD Biosciences, Oxford, UK). Cell concentrations were calculated from sample flow rate which was determined by addition of a known concentration of fluorescent latex beads as

an internal standard (Zubkov and Burkill, 2006). The detection limit was 10^3 cells per milliliter culture. For fluorescence microscopy formaldehyde fixed samples were filtered directly with a vacuum pump (Millipore, Billerica, MA, USA) under low, non-disruptive pressure ($<$ 5 mm Hg) and a 96-well blotting manifold (Bio-Dot, Bio-Rad, Munich, Germany) onto 4 mm polycarbonate filters with a pore size of 0.2 μm (GTTP, Millipore, Billerica, MA, USA). All filters were stored at –20 ℃ until further analyses. Filters were stained with either 1× SYBRGreen or 1 μg/ml DAPI and mounted on glass slides with Citifluor and VectaShield (4:1).

Sequencing and analysis of 16S rRNA and ITS

For colony PCR 1 mL culture was concentrated by centrifugation at 13,000x *g* for 10 min. The pellet was dissolved in 20 μL PCR water and subjected to three freeze-thaw cycles for cells lysis. Cell-free PCR water was used as control. PCR amplifications were performed at 96 ℃ for 4 min., 35 cycles of 96 ℃ for 1 min., 55 or 68 ℃ (depending on primer) for 1 min., 72 ℃ for 3 min. and 10 min. elongation at 72 ℃. The newly designed *Reinekea*-specific primer Rei732R (5' -TAT CAG CCC AGC AAG TC- 3') was based on the CARD-FISH probe Rei731 (Teeling et al., 2012), shortened by one nucleotide at the 3' end. The specificity of Rei732R was determined *in silico* with Probe Match (Cole et al., 2009) on the RDP homepage and with TestPrime (Klindworth et al., 2012) on the SILVA homepage (Pruesse et al., 2007). Gradient PCR with the primer pair 27F (5' -AGA GTT TGA TYM TGG CTC AG- 3') (Muyzer et al., 1995) and Rei732R revealed an optimal annealing temperature of 68 ℃. Intergenic spacer sequences were amplified and sequenced with the 16S rRNA primer 16S_1099 (5' -GYA ACG AGC GCA ACC C- 3') (Nossa et al., 2010) and the 23S rRNA primer L189R (5' -TAC TGA GAT GYT TMA RTT C- 3') (Yu and Mohn, 2001). Gradient PCR with the primer pair

16S_1099/L189R revealed an optimal annealing temperature of 46 ℃. The 16S rRNA gene of *Flavobacteria* was amplified and sequenced with the primer pair 27F/Fla-1489R as described previously (Hahnke and Harder, 2013). Sequencing reactions were performed using the ABI Dye Terminator technology according to the manufacturer's instructions (Applied Biosystems, Foster City, USA) with the following modifications: (a) an Applied Biosystems model 3130xl DNAsequencer (Applied Biosystems, Foster City, USA) was used for electrophoresis of the sequence reaction mixtures; (b) the 16S rRNA sequences were analyzed with Sequencing Analysis 5.2 (Applied Biosystems, Foster City, USA) and assembled with Sequencer 4.6 (Gene codes, Ann Arbor, MI, USA).

Phylogenetic affiliation

The initial phylogenetic affiliation was done using RDP (Cole et al., 2009). Obtained 16S rRNA sequences were aligned with the SINA aligner (Pruesse et al., 2007) and added to the tree by the parsimony method in ARB (Ludwig et al., 2004). Evolutionary distances were calculated to construct a phylogenetic consensus tree using neighbor-joining (Saitou and Nei, 1987) wizh a 0% and 40% base frequency filter.

Read recruitment

De-replicated reads from the metagenome libraries of 7 April 2009 (1,770,956 reads) and 14 April 2009 (4,062,242 reads) (Teeling et al., 2012) were mapped onto the draft sequenced genomes of the isolated strains. The mapping was carried out with the SSAHA2 (Ning et al., 2001) using default parameters. Coverage of a strain was computed by dividing the amount of bases aligned with the total bases of its draft genome, as described by Konstantinidis and DeLong (2008).

Subsequent isolation
To obtain pure cultures, selected enrichments were sub-cultured three times by diluting the microbial population in medium five times 1:10 and twelve times 1:2. The cultures were regularly examined for changes and impurities, including phenotypic characterizations, 16S rRNA gene amplifications, and CARD-FISH.

Transmission electron microscopy
For negative staining, bacterial cultures were adsorbed onto carbon film, washed in TE buffer (20 mM Tris/HCl, 1 mM EDTA, pH 6.9), stained with 4% (w/v) aqueous uranyl acetate (pH 4.5) according to the method of Valentin et al. (1968) and picked up with 300-mesh copper grids. After air-drying, samples were examined in a Zeiss EM109 transmission electron microscope (TEM) at an acceleration voltage of 80 kV and at calibrated magnifications.

Strain conservation All cultures were initially cryoconserved with liquid nitrogen at 80 ℃, frozen in HaHa and HaHa_100 medium supplemented with 30% glycerol (v/v). Isolates were maintained as viable cultures in HaHa and HaHa_100 medium at 4 ℃ and 11 ℃, and cryoconserved. A 10% inoculum was transferred into fresh medium every three months.

Nucleotide sequence accession numbers
All 16S rRNA, 16S-23S intergenic spacer (ITS) and proteorhodopsin sequences of this study were submitted using CDinFusion (Hankeln et al., 2011) and Sequin (http://www.ncbi.nlm.nih.gov/projects/Sequin/) and have been deposited in GenBank under accession numbers KF023483–KF023507 (16S rRNA), KF023508–KF023511 (proteorhodopsin) and KF023512–KF023514 (ITS).

3.4 Results

Bacterioplankton composition of the seawater sample

Isolation was carried out from sub-surface seawater sampled on 20 April 2010 near the North Sea island Helgoland in the German Bight. At that time, the bacterioplankton cell density accounted for 5.5 x 10^5 cells mL^{-1}. Based on microscopic cell counts obtained by fluorescence *in situ* hybridization *Bacteria* dominated 89% of the total picoplankton, comprising 31% *Alphaproteobacteria*, 22% *Gammaproteobacteria*, and 32% *Bacteroidetes* (Fig. 3.1). Members of the class *Flavobacteria* dominated 78% of the *Bacteroidetes*, with *Ulvibacter* (5.3×10^4 cells mL^{-1}; 9.9%), *Polaribacter* (4.2×10^4 cells mL^{-1}; 7.7%), and *Owenweeksia* (2.3×10^4 cells mL^{-1}; 4.2%) representing the most abundant clades, whereas the flavobacterial *Formosa* clade A and B, NS3, NS5 each represented less than 1% (5.5×10^3 cells mL^{-1}) of the total picoplankton cell counts. In contrast to *Flavobacteria*, members of the class *Cytophagia* accounted for less than 1% of the

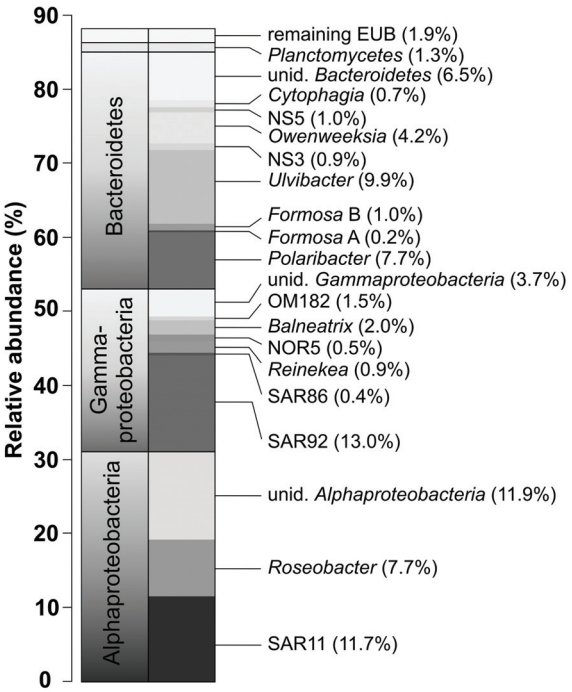


Figure 3.1 Relative abundance of bacterioplankton populations on 20 April 2010, as assessed by CARD-FISH: the left bar illustrates the bacteria composition on class (*Alphaproteobacteria* and *Gammaproteobacteria*) and phylum level (*Bacteroidetes* and *Planctomycetes*); the right bar represents specific clades as well as the unidentified remainder. Probes used are listed in supplementary (Tab. 3.1).

Table 3.1 Dilution cultures of the sampled seawater. The original microbial community accounted for 5.4 10^5 cells mL^{-1} on 20th April 2010 (Hel1) and for 3.2 10^6 cells mL^{-1} on 2nd September 2010 (Hel3).

Name of dilution cultures	Hel1_31	Hel1_32	Hel1_33	Hel3_A1
Dilution series	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Dilution after 100 μL inoculum	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Seawater inoculum (nL/sample)	100	10	10	0.1
Cells per 100 μL inoculum	54	5.4	0.5	0.3
No. of dilution cultures	50	100	140	100

Bacteroidetes. *Alphaproteobacteria* were represented for the most part by SAR11 (6.6×10^4 cells mL^{-1}; 12%) and *Roseobacter* clade members (4.4×10^4 cells mL^{-1}; 8%). The *Gammaproteobacteria* were dominated by members of the SAR92 clade (7.0×10^4 cells mL^{-1}). Other *Gammaproteobacteria* such as *Balneatrix*, *Reinekea*, and the OM182, NOR5 and SAR86 clades each accounted for less than 2% (1.0×10^4 cells mL^{-1}) of the total community.

Dilution cultivation of the bacterioplankton

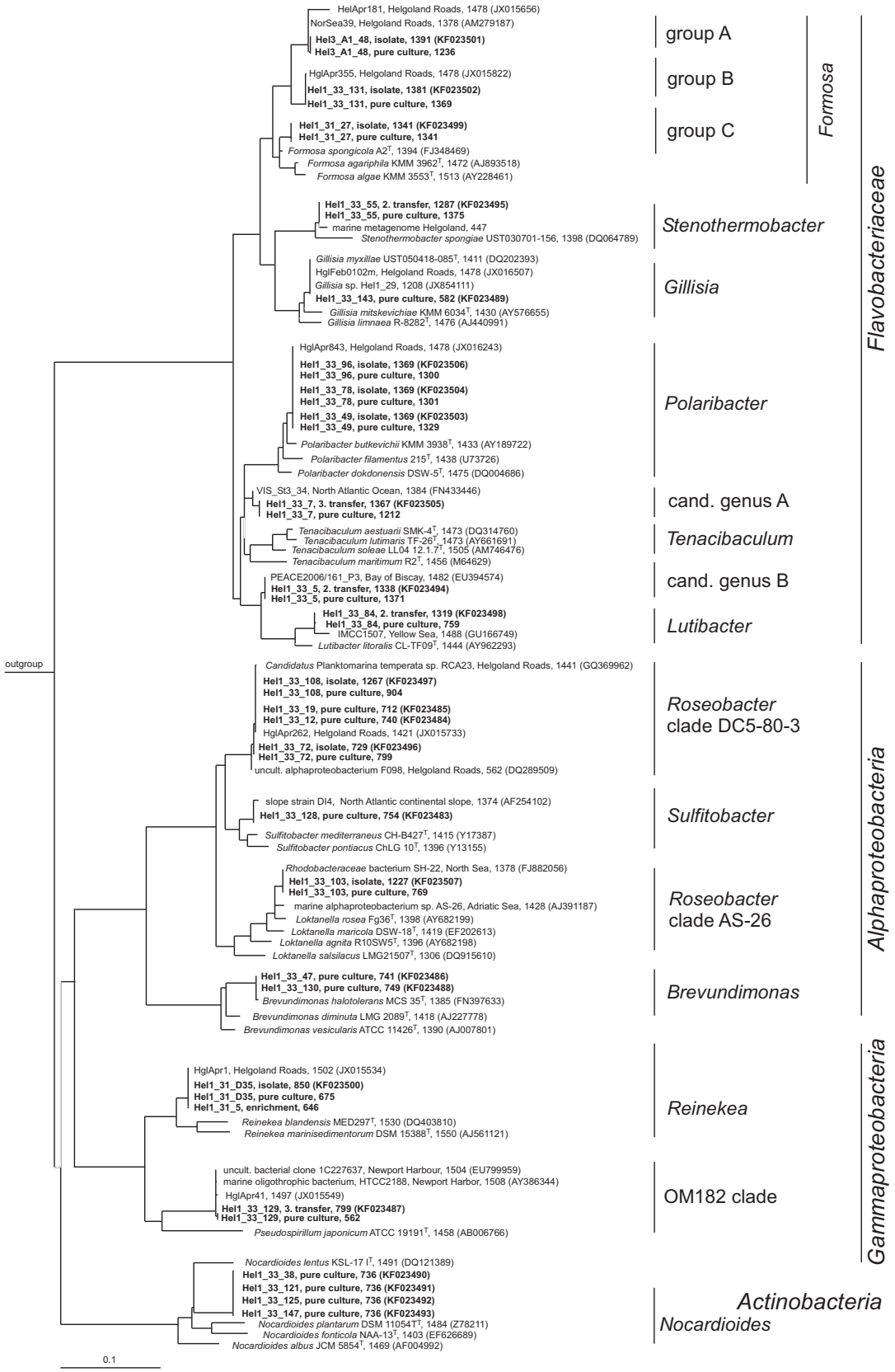
Two different approaches were undertaken, dilution cultivation of single cells and of small numbers of cells. For the cultivation of single cells, sampled seawater was diluted to a cell density of one cell per 200 μL (equivalent to 1 nL seawater; termed Hel1_33). Subsequently, aliquots of 100 μL (statistically 0.5 cells) of the diluted seawater were transferred to 140 cultivation tubes (Tab. 3.1). For the cultivation of small numbers of bacterioplankton cells, 100 dilution cultures were inoculated with approximately five cells per 100 μL (equivalent to 10 nL seawater; termed Hel1_32), and 50 dilution cultures with 50 cells per 100 μL (equivalent to 100 nL seawater; termed Hel1_31). After three months of incubation at 11 ℃ in the dark, growth to at least 10^3 cells mL^{-1} was detected by flow cytometry (suppl. Fig. 3.S3) in 24 of the 140 Hel1_33 dilution cultures, 74 of the 100 Hel1_32 dilution

cultures, and 50 of the 50 Hel1_31 dilution cultures. The average cell density was 5×10^6 cells mL^{-1}. Provided that a single cell was inoculated in the growth-positive Hel1_33 series, these represented pure cultures resulting from approx. 22 cell divisions. Indeed, 23 dilution cultures represented pure isolates and only dilution culture Hel1_33_69 was an enrichment of a mixed community, as determined by microscopy and flow cytometry (suppl. Fig. 3.S3).

Phylogenetic affiliations of the isolates

Based on 16S rRNA gene analysis (Fig. 3.2), nine cultures belonged to *Flavobacteriaceae*. One affiliated with 99.6% 16S rRNA gene identity with *Gillisia myxillae*, one with 99.0% 16S rRNA gene identity with *Nonlabens agnitus* and seven exhibited a 16S rRNA sequence identity of less than 98.6% to the next cultured strain and thus likely represented novel species (Stackebrandt and Ebers, 2006). These novel species included three closely related *Polaribacter* strains (mutual identity of 99.9%) which shared an identity of 97.5% with *P. butkevichii*, one *Formosa* strain with a 16S rRNA gene identity of 96% with *F. agariphila* and 97% with *F. algae*, and one *Lutibacter* strain with an identity of 96.7% with *L. litoralis*. Two strains represented novel candidate *Flavobacteriaceae* genera, one strain with a 16S rRNA gene identity of 93% to 95% with type strains of the genera *Tenacibaculum* and *Polaribacter*, and one with 93.0 to 95.2% with type strains of the genus *Lutibacter* (Fig. 3.2). The three *Polaribacter* cultures had identical 16S rRNA gene sequences as well as 99.9% identical 16S–23S

Figure 3.2 ***(facing page)*** Phylogenetic tree of isolates from the spring bloom at Helgoland in 2010 and clones of the spring bloom at Helgoland in 2009 (HglApr) or other seawater samples. Names of genera and clades are given to the right. Dilution cultures (pure culture) were transferred three times (transfer) to obtain isolates (isolate). A consensus tree was built with full-length sequences based on the neighbor-joining method, calculated without and with 40% *Bacteria* positional conservatory filters.


HelApr181, Helgoland Roads, 1478 (JX015656)
NorSea39, Helgoland Roads, 1378 (AM279187)
Hel3_A1_48, isolate, 1391 (KF023501)
Hel3_A1_48, pure culture, 1236
group A
HglApr355, Helgoland Roads, 1478 (JX015822)
Hel1_33_131, isolate, 1381 (KF023502)
Hel1_33_131, pure culture, 1369
group B
Hel1_31_27, isolate, 1341 (KF023499)
Hel1_31_27, pure culture, 1341
Formosa spongicola A2T, 1394 (FJ348469)
Formosa agariphila KMM 3962T, 1472 (AJ893518)
Formosa algae KMM 3553T, 1513 (AY228461)
group C
Formosa
Hel1_33_55, 2. transfer, 1287 (KF023495)
Hel1_33_55, pure culture, 1375
marine metagenome Helgoland, 447
Stenothermobacter spongiae UST030701-156, 1398 (DQ064789)
Stenothermobacter
Gillisia myxillae UST050418-085T, 1411 (DQ202393)
HglFeb0102m, Helgoland Roads, 1478 (JX016507)
Gillisia sp. Hel1_29, 1208 (JX854111)
Hel1_33_143, pure culture, 582 (KF023489)
Gillisia mitskevichiae KMM 6034T, 1430 (AY576655)
Gillisia limnaea R-8282T, 1476 (AJ440991)
Gillisia
HglApr843, Helgoland Roads, 1478 (JX016243)
Hel1_33_96, isolate, 1369 (KF023506)
Hel1_33_96, pure culture, 1300
Hel1_33_78, isolate, 1369 (KF023504)
Hel1_33_78, pure culture, 1301
Hel1_33_49, isolate, 1369 (KF023503)
Hel1_33_49, pure culture, 1329
Polaribacter butkevichii KMM 3938T, 1433 (AY189722)
Polaribacter filamentus 215T, 1438 (U73726)
Polaribacter dokdonensis DSW-5T, 1475 (DQ004686)
Polaribacter
VIS_St3_34, North Atlantic Ocean, 1384 (FN433446)
Hel1_33_7, 3. transfer, 1367 (KF023505)
Hel1_33_7, pure culture, 1212
cand. genus A
Tenacibaculum aestuarii SMK-4T, 1473 (DQ314760)
Tenacibaculum lutimaris TF-26T, 1473 (AY661691)
Tenacibaculum soleae LL04 12.1.7T, 1505 (AM746476)
Tenacibaculum maritimum R2T, 1456 (M64629)
Tenacibaculum
PEACE2006/161_P3, Bay of Biscay, 1482 (EU394574)
Hel1_33_5, 2. transfer, 1338 (KF023494)
Hel1_33_5, pure culture, 1371
cand. genus B
Hel1_33_84, 2. transfer, 1319 (KF023498)
Hel1_33_84, pure culture, 759
IMCC1507, Yellow Sea, 1488 (GU166749)
Lutibacter litoralis CL-TF09T, 1444 (AY962293)
Lutibacter
Flavobacteriaceae
outgroup
Candidatus Planktomarina temperata sp. RCA23, Helgoland Roads, 1441 (GQ369962)
Hel1_33_108, isolate, 1267 (KF023497)
Hel1_33_108, pure culture, 904
Hel1_33_19, pure culture, 712 (KF023485)
Hel1_33_12, pure culture, 740 (KF023484)
HglApr262, Helgoland Roads, 1421 (JX015733)
Hel1_33_72, isolate, 729 (KF023496)
Hel1_33_72, pure culture, 799
uncult. alphaproteobacterium F098, Helgoland Roads, 562 (DQ289509)
Roseobacter clade DC5-80-3
slope strain DI4, North Atlantic continental slope, 1374 (AF254102)
Hel1_33_128, pure culture, 754 (KF023483)
Sulfitobacter mediterraneus CH-B427T, 1415 (Y17387)
Sulfitobacter pontiacus ChLG 10T, 1396 (Y13155)
Sulfitobacter
Rhodobacteraceae bacterium SH-22, North Sea, 1378 (FJ882056)
Hel1_33_103, isolate, 1227 (KF023507)
Hel1_33_103, pure culture, 769
marine alphaproteobacterium sp. AS-26, Adriatic Sea, 1428 (AJ391187)
Loktanella rosea Fg36T, 1398 (AY682199)
Loktanella maricola DSW-18T, 1419 (EF202613)
Loktanella agnita R10SW5T, 1396 (AY682198)
Loktanella salsilacus LMG21507T, 1306 (DQ915610)
Roseobacter clade AS-26
Hel1_33_47, pure culture, 741 (KF023486)
Hel1_33_130, pure culture, 749 (KF023488)
Brevundimonas halotolerans MCS 35T, 1385 (FN397633)
Brevundimonas diminuta LMG 2089T, 1418 (AJ227778)
Brevundimonas vesicularis ATCC 11426T, 1390 (AJ007801)
Brevundimonas
Alphaproteobacteria
HglApr1, Helgoland Roads, 1502 (JX015534)
Hel1_31_D35, isolate, 850 (KF023500)
Hel1_31_D35, pure culture, 675
Hel1_31_5, enrichment, 646
Reinekea blandensis MED297T, 1530 (DQ403810)
Reinekea marinisedimentorum DSM 15388T, 1550 (AJ561121)
Reinekea
uncult. bacterial clone 1C227637, Newport Harbour, 1504 (EU799959)
marine oligothrophic bacterium, HTCC2188, Newport Harbor, 1508 (AY386344)
HglApr41, 1497 (JX015549)
Hel1_33_129, 3. transfer, 799 (KF023487)
Hel1_33_129, pure culture, 562
Pseudospirillum japonicum ATCC 19191T, 1458 (AB006766)
OM182 clade
Gammaproteobacteria
Nocardioides lentus KSL-17 IT, 1491 (DQ121389)
Hel1_33_38, pure culture, 736 (KF023490)
Hel1_33_121, pure culture, 736 (KF023491)
Hel1_33_125, pure culture, 736 (KF023492)
Hel1_33_147, pure culture, 736 (KF023493)
Nocardioides plantarum DSM 11054TT, 1484 (Z78211)
Nocardioides fonticola NAA-13T, 1403 (EF626689)
Nocardioides albus JCM 5854T, 1469 (AF004992)
Nocardioides
Actinobacteria
0.1

rRNA internal transcribed spacer (ITS) and proteorhodopsin sequences (suppl. Fig. 3.S4), but were clearly distinguishable based on their distinct morphological characteristics (Fig. 3.3). Another pure culture affiliated with 99.9% 16S rRNA gene identity with the *Gammaproteobacteria* clade OM182 strain HTCC2188. Eight pure cultures were identified as *Alphaproteobacteria*, four strains of which affiliated with 99.9% 16S rRNA gene identity with *Roseobacter* sp. RCA23 of the *Roseobacter* DC5-80-3 clade, one strain with 99.1% 16S rRNA gene identity with *Loktanella rosea* and one strain with 97.2% 16S rRNA gene identity with *Sulfitobacter mediterraneus*.

Culturability

Culturability with our HaHa liquid medium (Tab. 3.2) was 35 ± 7% of total cell counts (DAPI positive cells). The culturability differed significantly between *Gammaproteobacteria* (6%; probe GAM42a), *Alphaproteobacteria* (35%; probe ALF968), and *Bacteroidetes* (38%; probe CF319a). In contrast, growth was neither observed on HaHa agar and 2216 agar. Agar or its components were excluded as growth inhibitors, whereas HEPES buffer affected the growth of all pure cultures considerably (suppl. *Influence of the HEPES agar* on page 156).

Targeted isolation of Reinekea and Formosa clade A

Pure cultures of *Reinekea* were not directly obtained from the single cell series (Hel1_33; Tab. 3.1). This was probably a result of the low *in situ* *Reinekea* cell numbers of less than 5.0×10^3 cells mL^{-1}. Therefore, enrichments from inoculations with less diluted seawater (Hel1_31 and Hel1_32; Tab. 3.1) were screened by PCR with the *Reinekea*-specific primer pair 27F and Rei732R. *Reinekea* was detected in four out of the 50 enrichments, which corresponds to a culturability of 17%. Based on CARD-FISH (*Reinekea*-specific probe REI731) the relative abundance of *Reinekea*

Table 3.2 Estimated culturability of bacterial clades from which dilution cultures were obtained. Cell counts of bacterioplankton populations were determined by CARD-FISH using the indicated probes. The culturability and number of expected pure cultures were estimated as described by Button and colleques (1993). ASE, average standard error. n.a., not applicable.

Taxon	Microbial populations CARD-FISH probe	%	cells/mL	Dilution cultivation Inocul. (nL)	No. of cultures	Positive cultures	Culturability estimated % (ASE)	Pure cultures expected n (ASE)
Total cell counts	DAPI	100	$5.4\ 10^5$	100	50	50	n.a.	n.a.
				10	100	74	25 (3)	35 (2)
				1	140	24	35 (7)	22 (4)
Bacteria	EUB338I-III	88.7	$4.8\ 10^5$	100	50	50	n.a.	n.a.
				10	100	74	28 (4)	35 (2)
				1	140	24	39 (8)	22 (4)
Alphaproteobacteria	ALF968	31.3	$1.7\ 10^5$	1	140	8	35 (12)	8 (3)
RCA clade	ROS537	7.7	$4.2\ 10^4$	1	140	6	100 (43)	6 (2)
Gammaproteobacteria	GAM42a	22.0	$1.2\ 10^5$	1	140	1	1 (1)	1 (1)
OM182 clade	OM182_707	1.2	$8.1\ 10^3$	1	140	1	89 (9)	1 (1)
Reinekea	REI731	0.9	$5.5\ 10^6$	100	50	4	17 (8)	4 (2)
				10	100	0	n.a.	n.a.
				1	150	0	n.a.	n.a.
Bacteroidetes	CF319a	32.2	$1.7\ 10^5$	100	50	43	11 (2)	14 (2)
				10	100	47	37 (5)	34 (2)
				1	140	9	38 (13)	9 (3)
Polaribacter	POL740	7.7	$4.2\ 10^4$	1	140	3	52 (29)	3 (2)
Formosa clade A	FORM-181A	2.2	$2.7\ 10^4$	1	140	3	100 (8)	3 (1)
Formosa clade B	FORM-181B	1.0	$5.5\ 10^6$	1	140	1	100 (13)	1 (1)

was between 18% and 35% within the enrichments of approx. 3×10^6 cells per milliliter. From these, secondary dilution cultures were inoculated with theoretically two *Reinekea* cells from the two enrichments Hel1_31_5 and Hel1_31_27 (consecutive dilution cultures). *Reinekea* cells were detected by PCR in the dilution culture Hel1_31_D35 (D35, 35th tube of the 10^4 dilution). The purity of this culture was confirmed by microscopy and CARD-FISH. The strain affiliated with 96.4% 16S rRNA gene identity with *Reinekea blandensis* MED297^T (Pinhassi et al., 2007), and 95.4% with *Reinekea marinisedimentorum* KMM 3655^T (Romanenko et al., 2004) (Fig. 3.2). Consecutive dilution cultures of the enrichment Hel1_31_27 were negative for *Reinekea* cells, but yielded three *Formosa* strains (*Formosa* clade C) with a mutual 16S rRNA gene identity of 99.9% and 96% to 97% with *Formosa agariphila* DSM 15362^T (Nedashkovskaya et al., 2006), *Formosa algae* KMM 3553^T (Ivanova et al., 2004) and the isolate *Formosa* sp. Hel1_33_131 (*Formosa* clade B).

Dilution cultures of the *Formosa* clade A were not obtained from the seawater in spring 2010, probably because of the low *in situ* cell numbers of the *Formosa* clade A of less than 1.1×10^3 cells mL^{-1}. On 2 September 2010, the *Formosa* clade A accounted for 2.7×10^4 cells mL^{-1} (2.2% of the total picoplankton) in the seawater of Helgoland. Therefore, this seawater was diluted to a cell density of one cell per 300 μL (equivalent to 1 nL seawater; termed Hel3_A1), and aliquots of 100 μL (statistically 0.3 cells) of the diluted seawater were transferred to 100 cultivation tubes (Tab. 3.1). Growth was observed in 50 of the 100 Hel3_A1 dilution cultures, among them 16S rRNA sequences of the *Formosa* clade A (dilution culture Hel3_A1_48). The strain affiliated with 96% to 97% 16S rRNA gene identity with *Formosa agariphila* DSM 15362^T, *Formosa algae* KMM 3553^T, the isolate *Formosa* sp. Hel1_33_131 (*Formosa* clade B), and *Formosa* sp. Hel1_31_27 (*Formosa* clade C). *Formosa* group specific CARD-FISH of cultures with the probe Form181A and Form181 corroborated the purity of the culture and affiliation with the *Formosa* clade A and B.

Environmental relevance

Strains isolated from the 20 April 2010 seawater sample had 16S rRNA gene sequence identities of more than 99.8% with clone library sequences from the preceding spring phytoplankton bloom in 2009, such as strains of *Polaribacter*, *Gillisia*, *Reinekea*, the *Formosa* clade B, the OM182 clade, and the *Roseobacter* clade associated (RCA) lineage (Tab. 3.3). In contrast, 16S rRNA sequences of *Formosa* clade A and C, *Lutibacter*, *Loktanella*, *Sulfitobacter*, and *Brevundimonas* cultures were not found in the clone library, but close relatives with more than 97.8% 16S rRNA gene identity were present in other clone libraries from Helgoland seawater or other marine habitats (Tab. 3.3).

Draft genome sequencing of *Formosa* sp. Hel3_A1_48 (*Formosa* clade A),

Table 3.3 Phylogenetic affiliation of obtained pure cultures and isolates to next relative type strains or 16S rRNA sequences of uncultured marine bacteria. HglApr and HglFeb sequences are 16S rRNA gene sequences of bacterioplankton clone library of the spring phytoplankton bloom at Helgoland in 2009

Taxonomy	Isolate	Inoculum	Next relative type strains	Identity (%)	Next relative unc. sequence	Identity (%)	Accession number
Flavobacteriaceae							
Polaribacter	Hel1_33_49	1 nL	*P. butkevichii*	97.5	HglApr843	99.9	JX016243
	Hel1_33_78	1 nL					
	Hel1_33_96	1 nL					
Formosa	Hel3_A1_48	1 nL	*F. agariphila*	96.1	NorSea39	99.6	AM279187
	Hel1_33_131	1 nL	*F. agariphila*	96.0	HglApr355	100	JX015822
	Hel1_31_27	100 nL	*F. spongicola*	97.6	S26-122	97.6	EU287422
Gillisia	Hel1_33_143	1 nL	*G. myxillae*	96.5	HglFeb0102m	99.8	JX016507
					Gillisia sp. Hel1_29[a]	99.9	JX854111
Lutibacter	Hel1_33_84	1 nL	*L. litoralis*	96.7	IMCC1507	97.8	GU166749
Nonlabens	Hel1_33_55	1 nL	*N. agnitus*	99.0			HM475136
gen. nov. HelA	Hel1_33_7	1 nL	*Polaribacter* sp.	93-95.3	VIS_St3_34	98.4	FN433446
gen. nov. HelB	Hel1_33_5	1 nL	*Lutibacter* sp.	93-95.2	PEACE2006/161_P3	98.8	EU394574
Gammaproteobacteria							
Reinekea	Hel1_31_D35	100 nL	*Reinekea* sp.	95.4-96.4	HglApr1	99.9	JX015534
OM182 clade	Hel1_33_129	1 nL	OM182 strain HTCC2188	99.9	HglApr41	99.9	JX015549
Alphaproteobacteria							
RCA lineage	Hel1_33_72	1 nL	*Octadecabacter*	95.5	HglApr262	99.9	JX015733
	Hel1_33_108	1 nL	*antarcticus*		F098	99.9	DQ289509
	Hel1_33_12	1 nL			RCA23[a]	99.9	GQ369962
	Hel1_33_19	1 nL					
Loktanella	Hel1_33_103	1 nL	*L. rosea*	99.1	SH22-2a	99.9	FJ882056
Sulfitobacter	Hel1_33_128	1 nL	*S. mediterraneus*	97.2	slope strain DI4[a]	99.7	AF254102
Brevundimonas	Hel1_33_47	1 nL	*B. halotolerans*	99.7			FN397633
	Hel1_33_130	1 nL					
Actinobacteria							
Nocardia	Hel1_33_38	1 nL	*N. plantarum*	95.2	HF500_03E09	100	EU361007
	Hel1_33_121	1 nL					
	Hel1_33_125	1 nL					
	Hel1_33_147	1 nL					

[a]strains of cultured marine bacteria

Formosa sp. Hel1_33_131 (*Formosa* clade B), *Reinekea* sp. Hel1_31_D35, and *Polaribacter* sp. Hel1_33_49, and subsequent read-recruitment of metagenomes obtained during of the spring bloom of 2009 also indicated that the isolates were indeed representative of the pelagic bacterioplankton community in spring 2009 (Tab. 3.4). Metagenomic reads with $\geqslant$ 95% nucleotide identity covered 94% of the *Formosa* sp. Hel1_33_131, and 90% of the *Reinekea* sp. Hel1_31_D35 draft-genomes, and recruited 3.75% (66,441 reads) and 4.44% (180,245 reads) of the reads, respectively. This suggests that these strains represented discrete populations during the spring phytoplankton bloom in 2009. Lower numbers of metagenomic reads were

Table 3.4 Coverage of isolate draft genomes by metagenome reads. Mapping of dereplicated metagenomic reads from 7 April 2009 onto the genomes of *Formosa* and from 14 April 2009 onto the genomes of *Reinekea* and *Polaribacter*.

	Genome Size (bp)	Contigs	Cumulative coverage by metagenome reads of ⩾ 95% nucleotide identity					
Formosa sp. Hel3_A1_48	2,050,062	3	0.05	0.08	0.13	0.17	0.18	0.18
Formosa sp. Hel1_33_131	2,780,744	1	0.39	1.10	3.00	7.24	7.55	7.56
Reinekea sp. Hel1_31_D35	3,713,075	79	0.40	1.01	2.07	4.41	10.3	15.3
Polaribacter sp. Hel1_33_49	3,051,453	31	0.05	0.11	0.23	0.35	0.36	0.36

recruited by draft-genomes of *Formosa* sp. Hel3_A1_48 (0.12%; 4,930) and *Polaribacter* sp. Hel1_33_49 (0.07%; 1,174), probably because the *Polaribacter* and *Formosa* clade A populations during the 2009 spring phytoplankton bloom 2009 were of lesser genomic coherence (Tab. 3.4). This was also reflected in the metagenomic reads covering only 50% of the *Polaribacter* sp. Hel1_33_49 and 10% of the *Formosa* sp. Hel3_A1_48 draft genomes.

3.5 Discussion

Our approach combined the artificial seawater (ASW) medium of Widdel and Bak (1992), with the modifications introduced by Hahnke and Harder (2013), and the dilution cultivation approach originally introduced by Button and colleagues (1993). The observed culturability of 35% is in the same range as reported for novel optimized cultivation techniques, such as 40% from soil (Kaeberlein et al., 2002), 37% from freshwater (Bussmann et al., 2001), and 20% (Connon and Giovannoni, 2002) or 50% (Button et al., 1993) from seawater. Our assessment of culturability based on total cell counts likely underestimates, since up to 10% of the bacterioplankton was likely dead or dormant (Ouverney and Fuhrman, 1999; Campbell et al., 2011). These cultivations in liquid HaHa medium resulted in a more than 100 times higher culturability as HaHa agar (Hahnke and Harder, 2013)

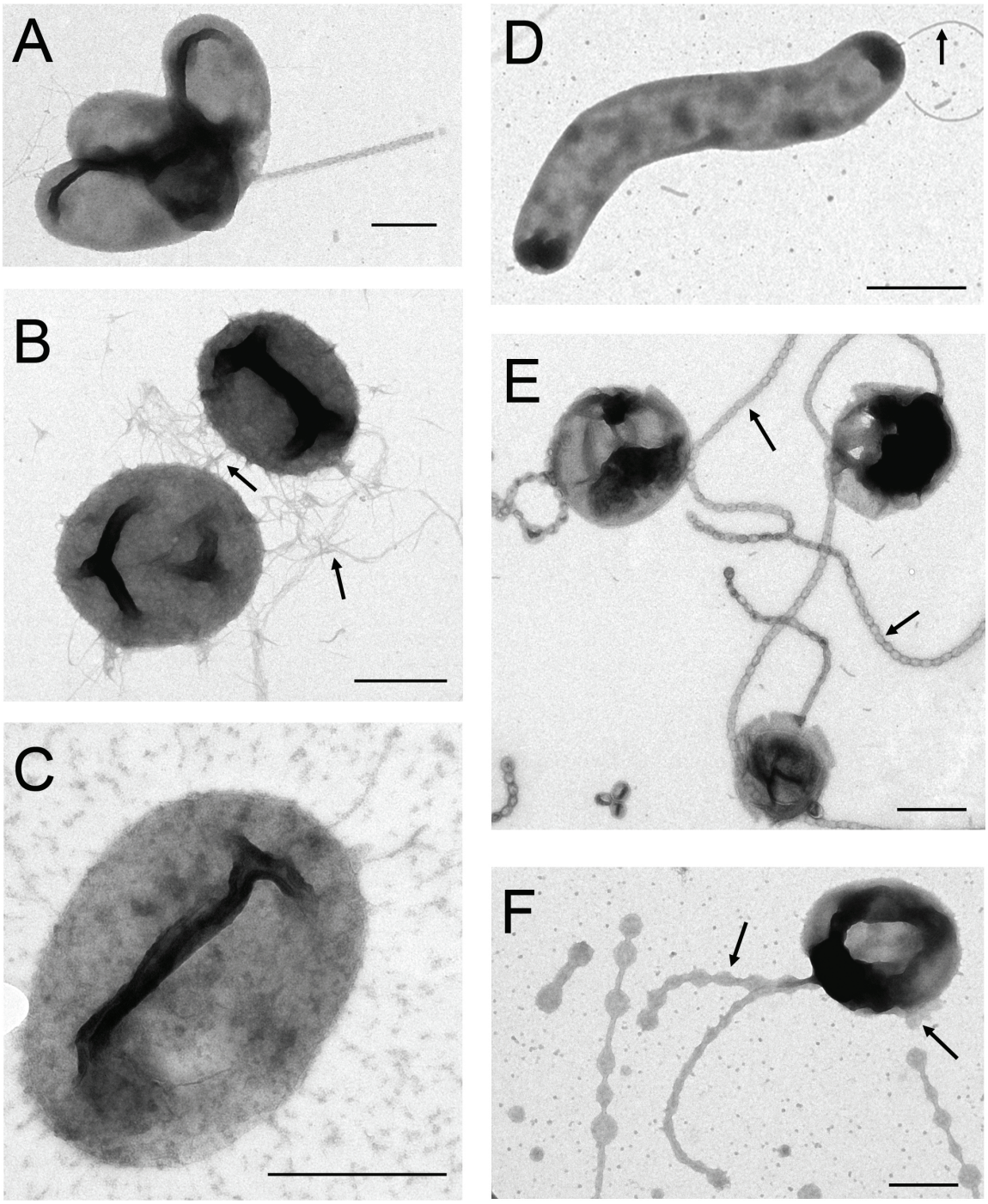


Figure 3.3 Cellular morphologies of marine bacteria documented by transmission electron microscopy images of **(A)** ***Polaribacter*** **sp. Hel1_33_49**, **(B)** ***Polaribacter*** **sp. Hel1_33_78**, **(C)** ***Polaribacter*** **sp. Hel1_33_96**, **(D)** ***Reinekea*** **sp. Hel1_31_D35**, **(E)** ***Formosa*** **sp. Hel3_A1_48** of the *Formosa* clade A, and **(F)** ***Formosa*** **sp. Hel1_33_131** of the *Formosa* clade B. Arrows indicate (B) fibers on the cell surface and in the surrounding, (D) a peritrichous flagellum, and (E, F) cellular appendages like strings of pearls forming a cobweb that connects the cells. Bar: 0.5 μm.

with the same samples. It has been shown before that many of the marine bacteria obtained by dilution cultivation cannot be grown on agar plates (Connon and Giovannoni, 2002; Kaeberlein et al., 2002). This inability to grow on agar plates as well as on HEPES-buffered medium might constitute reasons for the low cultivation efficiencies in previous studies (Eilers et al., 2000, 2001; Sapp et al., 2007a; Hahnke and Harder, 2013).

We observed a much lower culturability of *Gammaproteobacteria* than for *Alphaproteobacteria* and *Flavobacteria*. This demonstrates that the HaHa medium is unlikely to suffer from an over-representation of *Gammaproteobacteria*, known as cultivation-induced *Gammaproteobacteria* shift (Fuchs et al., 2000; Massana and Jürgens, 2003) which corroborates previous results with this medium (Hahnke and Harder, 2013). In addition, our cultivation approach allowed to isolate strains of *Formosa*, *Polaribacter*, *Reinekea*, *Lutibacter*, and the OM182 clade that were not brought into culture by previous cultivation studies of the North Sea (Eilers et al., 2000, 2001; Sapp et al., 2007a; Stevens et al., 2009; Hahnke and Harder, 2013). These previous failures are remarkable, since the cultivated *Formosa*, *Polaribacter*, and *Roseobacter* strains reached abundances of one to eight percent in spring 2010, and even higher abundances in spring 2009 (Teeling et al., 2012). On the other hand, we were not able to cultivate representatives of all *in situ* abundant taxa determined by CARD-FISH, such as *Ulvibacter*, SAR11, and SAR92. This suggests that the HaHa medium did not allow for growth of oligotrophic bacteria like SAR11 (Carini et al., 2012). However, we were able to directly cultivate a close relative of the strain HTCC2188 from the Pacific Ocean (Cho and Giovannoni, 2004). This strain was cultivated in sterilized oligotrophic seawater and belonged to the marine *Gammaproteobacteria* clade OM182 which consists of obligate oligotrophic bacteria. Hence, our approach facilitated the cultivation of oligotrophic bacteria. Further factors, like reactivated prophages, viral

infections, signal molecules and substrate requirements that influence the culturability of marine bacteria have been elaborately discussed elsewhere (Zengler, 2009; Overmann, 2010; Lennon and Jones, 2011).

Sañudo-Wilhelmy et al. proposed that auxotrophy for at least one B vitamin is common for both eukaryotic phytoplankton and bacterioplankton (Sañudo-Wilhelmy et al., 2012). This was for example shown for the vitamins B_1, B_5, B_7, and B_{12} in '*Candidatus* Pelagibacter ubique' HTCC1062 (Giovannoni et al., 2005). Indeed, the *Reinekea* and the *Formosa* clade A strains required vitamins for isolation. *In situ* sources of vitamins are algae and bacteria, but also influxes from rivers in coastal ecosystems (Gobler et al., 2007), suggesting a selection of bacterial populations whose vitamin needs match natural vitamin availability (Sañudo-Wilhelmy et al., 2012). The *Reinekea* population increased rapidly after a shift in the phytoplankton composition and collapsed two weeks later (Teeling et al., 2012) which might have resulted from a phytoplankton or bacterioplankton-induced release of vitamins or substrates.

Co-cultivation

Dilution cultivation (i.e. dilution to near extinction) allows both, cultivations starting from single cells and from bacterial communities consisting of only a few cells (Button et al., 1993). Cultivation of single cells circumvents the competition of slow growing and fast growing, opportunistic bacteria, whereas co-cultivation enables helper organisms to promote growth of otherwise non-culturable bacteria (D'Onofrio et al., 2010). For example, detoxification of hydrogen peroxide by helper bacteria can reduce the oxidative stress of beneficiaries (Morris et al., 2012). The evolutionary adaptation towards helping and benefiting bacteria that results in an increased overall fitness of the entire bacterial community has been discussed as the 'Black Queen hypothesis' (Morris et al., 2012).

Interestingly, we obtained the two *Reinekea*-positive enrichments, Hel1_31_5 and Hel1_31_27, but after the consecutive dilution cultivation of both enrichments, *Reinekea* was detected only in dilution cultures of the enrichment Hel1_31_5. This suggests that the dilution influenced the bacterial community in Hel1_31_27 in a way that prohibited growth of *Reinekea*, whereas it influenced the bacterial community in Hel1_31_5 in a way that promoted growth of *Reinekea*. The bacterial community of the latter consisted mainly of *Polaribacter*, and co-occurrence of *Reinekea* and *Polaribacter* was exactly what was observed *in situ* during the spring phytoplankton bloom in 2009 (Teeling et al., 2012). Therefore, a helper-beneficiary relationship of the *Polaribacter* and *Reinekea* is likely and will be subject of future studies.

Ecological relevance of the isolates

Our novel pure cultures were closely related (>99.9% 16S rRNA identity) to so far uncultured marine bacteria from the 2009 spring phytoplankton bloom in the German Bight of the North Sea (Teeling et al., 2012), bacterioplankton from Helgoland sampled at other occasions (Alonso et al., 2007; Sapp et al., 2007b), or other marine clone libraries (Pham et al., 2008; Li et al., 2009). However, a 16S rRNA identity of 98.7% to 100% alone is an insufficient criterion for whether or not these isolates belong to the afore-seen species (Rosselló-Mora and Amann, 2001; Yarza et al., 2010). As additional criterion, we amplified and sequenced the proteorhodopsin sequences of our *Flavobacteriaceae* isolates, which were 100% identical to proteorhodopsin sequences of phytoplankton bloom-associated bacterial communities from Helgoland in spring 2009 (Teeling et al., 2012) and summer 2006 (Riedel et al., 2010). Moreover, Riedel and colleagues affiliated the proteorhodopsin sequences Hel31 to *Flavobacteriaceae*, but could not provide a genus (Riedel et al., 2010). Here we suggest that this proteorhodopsin

belongs to the *Formosa* clade A within the genus *Formosa* (supplementary material). Interestingly, our strain Hel1_33_7 (candidate *Flavobacteriaceae* genus) had a proteorhodopsin sequence with 99.9% sequence identity to the proteorhodopsin clones NA13_R15_12 and NA11_R15_8 from the North Atlantic Ocean (Sabehi et al., 2005). This cluster of proteorhodopsin sequences was also previously phylogenetically uncharacterized (Sabehi et al., 2005). Proteorhodopsin sequences have been estimated to be present in 13% of the bacterioplankton in the photic zone (Sabehi et al., 2005) and are believed to be frequent subjects of lateral gene transfer (Frigaard et al., 2006; Sharma et al., 2006; Riedel et al., 2010) which of course might have affected our taxonomic proteorhodopsin affiliations.

Konstantinidis and Tiedje analyzed complete genomes as well as environmental metagenomes and proposed an average nucleotide identity (ANI) of 94% and a genomic sequence divergence of 5–6% as criteria for ecologically coherent species (Konstantinidis and Tiedje, 2005; Konstantinidis and DeLong, 2008). The genomes of our *Reinekea* and *Formosa* clade B isolates were covered by 90–94% with metagenomic sequences with $\geqslant$ 95% nucleotide identity. Both, the isolates and the metagenomes were derived from samples of the same habitat that were taken a year apart during spring phytoplankton blooms. Consequently, *Reinekea* sp. Hel1_31_D35 and *Formosa* sp. Hel1_33_131 formed discrete bacterioplankton populations (e.g. populations with a high level of genomic coherence) during spring 2009. The isolates of *Polaribacter* and the *Formosa* clade A were not part of such discrete populations, but with draft genome coverage of 50% and 10% with metagenomic sequences with $\geqslant$ 95% nucleotide identity, respectively, still similar to populations observed in 2009.

Members of the *Bacteroidetes* play a pivotal role in the degradation of complex organic matter (Kirchman, 2002; Schauer et al., 2008; Gómez-Pereira et al., 2012; Teeling et al., 2012). *Flavobacteria* are non-motile

or move by gliding (Bernardet, 2010) and many form biofilms, which enables them to colonize surfaces including living phytoplankton and allows them to stay close to the substratum (Reichenbach, 1981; Gómez-Pereira et al., 2012). This agrees with the observed strong attachment and aggregate formation of the *Polaribacter* isolates, that was mediated by fibers on their cell surfaces (Fig. 3.3). Likewise, formation of three-dimensional polysaccharides nets as observed for the *Formosa* isolates could increase the viscosity of the surrounding medium (Fig. 3.3) and might participate in particle formation, as shown for *Lentisphaera araneosa* (Cho and Giovannoni, 2004).

Conclusions

Our cultivation approach enabled a high-throughput cultivation in a dedicated low nutrient medium with little equipment. Representatives of so far uncultivated marine bacteria were successfully brought into pure culture directly from the seawater. The obtained isolates represented ecologically relevant and coherent species in the bacterioplankton during a phytoplankton bloom in the German Bight of the North Sea.

The represented taxa reached a cumulative abundance of 50% of total cell counts or successively accounted each for 8% to 27% of the bacterioplankton during the spring phytoplankton bloom at Helgoland in 2009 (Teeling et al., 2012). Hence, our isolates represent taxa of ecological relevance. Further physiological studies on these isolates will help to understand their specific ecological roles in the process of phytoplankton biomass decomposition and thereby elucidate the factors that define their ecological niches.

3.6 Acknowledgments

We acknowledge all members of the Microbial Interactions in Marine Systems project (MIMAS) project for data provision. We are grateful to Jörg Peplies for comparing the 16S rRNA sequences of our isolates with the classified MIMAS 16S metagenomes and 16S tags, and Erhard Rhiel from the Institute of Chemistry and Biology of the Marine Environment (ICBM) in Oldenburg for electron microscopy and image analysis. We thank Jörg Wulf, Greta Reintjes, and Maria Grünberg for CARD-FISH analyses. We thank Dr. Gunnar Gerdts, Hilke Döpke and the Biological Institut Helgoland (BAH) for laboratories access on Helgoland. This research was funded by the Max Planck Society.

3.7 Supporting Information

Pure culture isolation of marine heterotrophic bacteria benefiting from a coastal diatom bloom

Richard L. Hahnke, Christin M. Bennke, Bernhard M. Fuchs, Alexander J. Mann, Hanno Teeling, Rudolf Amann and Jens Harder

Table 3.S1 Probes, competitors and helpers used in this study. FA, Formamide concentration.

Probe name	Target group	Probe sequence (5' → 3')	FA (%)	Reference
EUB338-I	*Bacteria*	GCTGCCTCCCGTAGGAGT	35	Amann et al. 1990
EUB338-II	Supplement to EUB338	GCAGCCACCCGTAGGTGT	35	Daims et al. 1999
EUB338-III	Supplement to EUB338	GCTGCCACCCGTAGGTGT	35	Daims et al. 1999
NON338	Control	ACTCCTACGGGAGGCAGC	35	Wallner et al. 1993
ALF968	*Alphaproteobacteria*	GGTAAGGTTCTGCGCGTT	35	Neef 1997
SAR11-441R	SAR11-clade	TACAGTCATTTTCTTCCCCGAC	25	Rappé et al. 2002
ROS537	*Roseobacter* clade	CAACGCTAACCCCCTCC	35	Eilers et al. 2001
GAM42a	*Gammaproteobacteria*	GCCTTCCCACATCGTTT	35	Manz et al. 1992
Beta42a-Comp	Competitor for GAM42a	GCCTTCCCACTTCGTTT		Manz et al. 1992
SAR86-1245	SAR86-clade	TTAGCGTCCGTCTGTAT	35	Zubkov et al. 2001
SAR86-1245h3	Helper for SAR86-1245	GGATTRGCACCACCTCGCGGC		Zubkov et al. 2001
SAR86-1245h5	Helper for SAR86-1245	CCATTGTAGCACGTGTGTAGC		Zubkov et al. 2001
SAR92-627	SAR92-clade	CAGACAGTTCTAACTGCAGTTCC	20	Stingl et al. 2007
REI731	*Reinekea*	TATCAGCCCAGCAAGTCG	20	Teeling et al. 2012
NOR5-730	NOR5/OM60-clade	TCGAGCCAGGAGGCCGCC	50	Eilers et al. 2001
NOR5-709h	Helper for NOR5-730	TTCGCCACYGGTATTCCTCCA		Yan et al. 2009
NOR5-659h	Helper for NOR5-730	GAATTCTACCTCCCTCTCYCG		Yan et al. 2009
BAL731	*Balneatrix*-clade	TATCAAGCCAGGGCGTGC	25	Kassabgy 2011
OM182-707	OM182-clade	CACCGGTATTCCTCAGAA	15	Kassabgy 2011
CF319a	*Bacteroidetes*	TGGTCCGTGTCTCAGTAC	35	Manz et al. 1996
POL740	*Polaribacter*-clade	CCCTCAGCGTCAGTACATACGT	35	Malmstrom et al. 2007
FORM181A	*Formosa* clade A	GATGCCACTCTAAGAGAC	25	Teeling et al. 2012
FORM181A_Comp	Competitor for FORM181A	GATGCCACTCTTAGAGAC		Teeling et al. 2012
FORM181B	*Formosa* clade B	GATGCCACTCTTAGAGAC	35	Bennke et al. 2013 in prep.
FORM181B_Comp	Competitor for FORM181B	GATGCCACTCTAAGAGAC		Bennke et al. 2013 in prep.

Continued on next page

Table 3.S1 (continued)

Probe name	Target group	Probe sequence (5' ARROW 3')	FA (%)	Reference
ULV995	*Ulvibacter*-clade I	TCCACGCCTGTCAGACTACA	35	Teeling et al. 2012
ULV995_Comp1	Competitor for ULV995	TCCACTCCTGTCAGACTACA		Teeling et al. 2012
ULV995_Comp2	Competitor for ULV995	TCCACCCCTGTCAGACTACA		Teeling et al. 2012
NS3a-840	NS3a-clade	CTTAGCCGCTCAGAACTCAAGG	35	Bennke et al. 2013 in prep.
NS3a-840_Comp1	Competitor for NS3a-840	CTTGGCCGCCCAGAACTCAAGG		Bennke et al. 2013 in prep.
NS3a-840_Comp2	Competitor for NS3a-840	CTTGGCCGCCCAGCACTCAAGG		Bennke et al. 2013 in prep.
NS3a-840_h1	Helper for NS3a-840	TYCCGAACAGCTAGTATCCATCGTT		Bennke et al. 2013 in prep.
NS3a-840_h2	Helper for NS3a-840	CCAGGTGGGATACTTATCACTTTCG		Bennke et al. 2013 in prep.
VIS6-814	*Owenweeksia*-clade	CAGCGAGTGATGATCGTT	25[a]	Gómez-Pereira et al. 2010
VIS6-814_comp	Competitor for VIS6-814	CAGCGAGTGATCATCGTT		Gómez-Pereira et al. 2010
VIS6-814_h1	Helper for VIS6-814	TACGGCGTGGACTACCAGGT		Bennke et al. 2013 in prep.
VIS6-814_h2	Helper for VIS6-814	CCGCYGACAGTATATCGCCAA		Bennke et al. 2013 in prep.
NS5/DE2-471	NS5-clade I	GTAAGTAGGTTTCTTCCTGTAT	25	Gómez-Pereira et al. 2012
NS5/DE2-471_comp1	Competitor for NS5/DE2-471	GTAAGTAGGTTTCTTCCTGTAGAAAA		Gómez-Pereira et al. 2012
NS5/DE2-471_comp2	Competitor for NS5/DE2-471	GTAAGTAGGTTTCTTCCTATAT		Gómez-Pereira et al. 2012
CYT-734	*Marinoscillum*-clade	CAGTTTCTGCCTAGTAAG	25	Gómez-Pereira et al. 2012
PLA46	*Planctomycetes*	GACTTGCATGCCTAATCC	30	Neef et al. 1998

[a] former formamide concentration was at 15%, together with helpers formamide concentration could be increased to 25%.

Table 3.S2 Composition of casamino acids, tryptone peptone and yeast extract as listed in the technical manual of BD Bionutrients™, BD Biosciences, Sparks, MD, USA.

Product Name (Bacto, DIFCO)		Casamino Acids	Peptone	Yeast Extract
Inorganic compounds				
Loss on drying	%	4.8	2.3	3.1
Ash	%	18.3	6.6	11.2
pH	1% solution	6.4	7.3	6.7
NaCl	%	12.1	0.0	0.1
Potassium	mg/g	4.1	3.3	32.0
Sodium	mg/g	88.1	33.9	4.9
Magnesium	μg/g	143.0	195.0	750.0
Calcium	μg/g	59.0	256.0	130.0
Iron	μg/g	1.3	23.0	55.3
Chloride	μg/g	6.7	0.1	0.4
Sulfate	μg/g	0.6	0.3	0.1
Phosphate	μg/g	2.6	2.6	3.3
Total nitrogen (TN)	%	10.8	13.3	10.9
Amino nitrogen (ANT)	%	9.4	5.3	6.0
ANT/TN	%	0.9	0.4	0.6
Total carbohydrate	mg/g	0.0	4.3	163.3
Amino acids				
positive charged AA at pH 7.4				
Arginine (R)	% free	2.4	2.2	1.4
	% total	2.5	5.0	2.6
Histidine (H)	% free	0.2	0.5	0.4
	% total	0.8	1.9	1.3
Lysine (K)	% free	2.1	5.5	1.9
	% total	5.2	6.2	4.6
nagative charged AA at pH 7.4				
Aspartic Acid (D)	% free	0.7	0.4	1.6
	% total	2.4	5.2	5.3
Glutamic Acid (E)	% free	15.1	1.4	6.6
	% total	15.9	15.1	9.4
polar uncharged AA				
Asparagine (N)	% free	0.0	0.6	1.0
Glutamine (Q)	% free	0.0	0.1	0.2
Serine (S)	% free	0.4	0.7	1.3
	% total	2.1	2.2	1.6
Threonine (T)	% free	0.5	0.7	1.1
	% total	1.5	1.8	1.6
special cases				
Cystine (C)	% free	0.1	0.3	0.2
Glycine (G)	% free	1.4	0.2	1.0
	% total	1.4	1.7	3.0
Proline (P)	% free	7.5	0.2	0.8
	% total	8.0	6.6	2.0
hydrophobic side chain				
Alanine (A)	% free	3.0	1.0	4.4
	% total	3.0	3.2	5.6
Isoleucine (I)	% free	3.1	1.3	1.8
	% total	4.0	5.5	3.0
Leucine (L)	% free	4.6	4.8	3.0
	% total	5.0	7.5	4.1
Methionine (M)	% free	1.4	1.0	0.6
	% total	1.4	2.1	0.8
Phenylalanine (F)	% free	3.4	3.0	2.0
	% total	3.6	5.2	2.6
Tryptophan (W)	% free	0.0	0.8	0.5
Tyrosine (Y)	% free	0.4	0.5	0.8
	% total	0.4	1.3	1.2
Valine (V)	% free	4.7	1.7	2.2
	% total	5.6	5.9	3.5

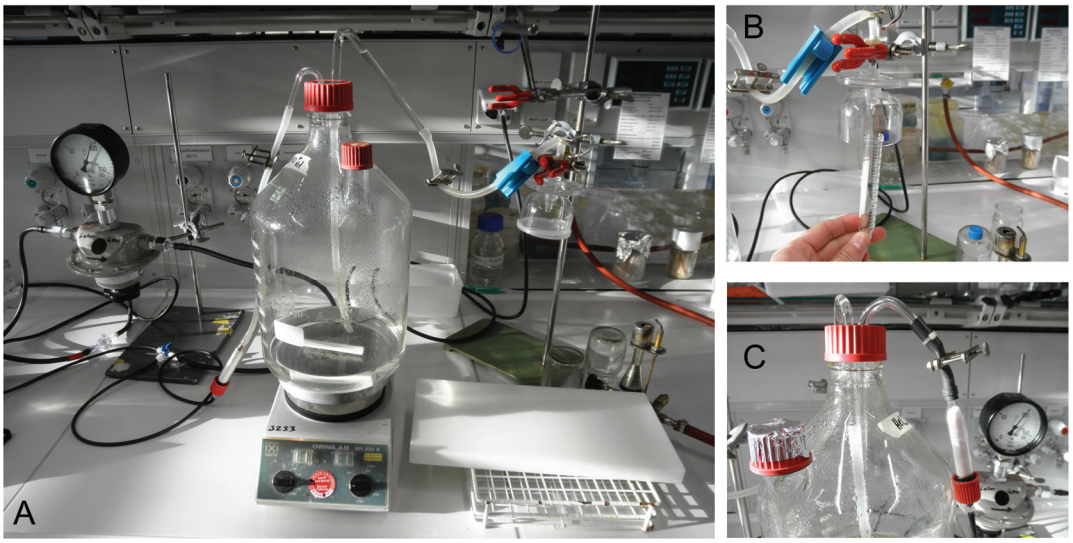


Figure 3.S1 Setup of the modified Widdel flask for medium preparation. (A) Complete setup. (B) Hose clamp, sterile filter, and a sterile bell to fill the medium into polystyrene tubes. (C) Pressure reducer, sterile cotton wool filter, and glass tubes to produce an overpressure of 100 mbar in the headspace of the medium flask.

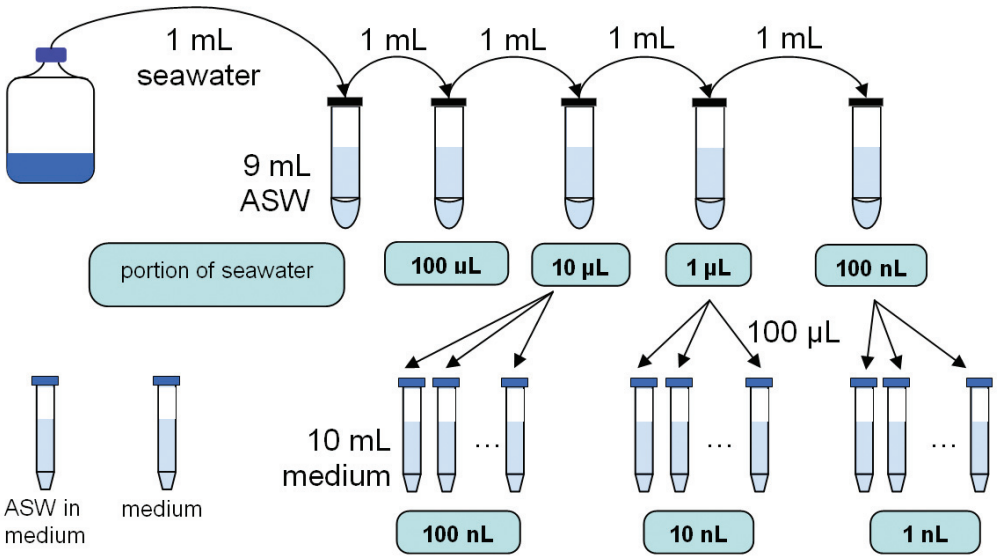


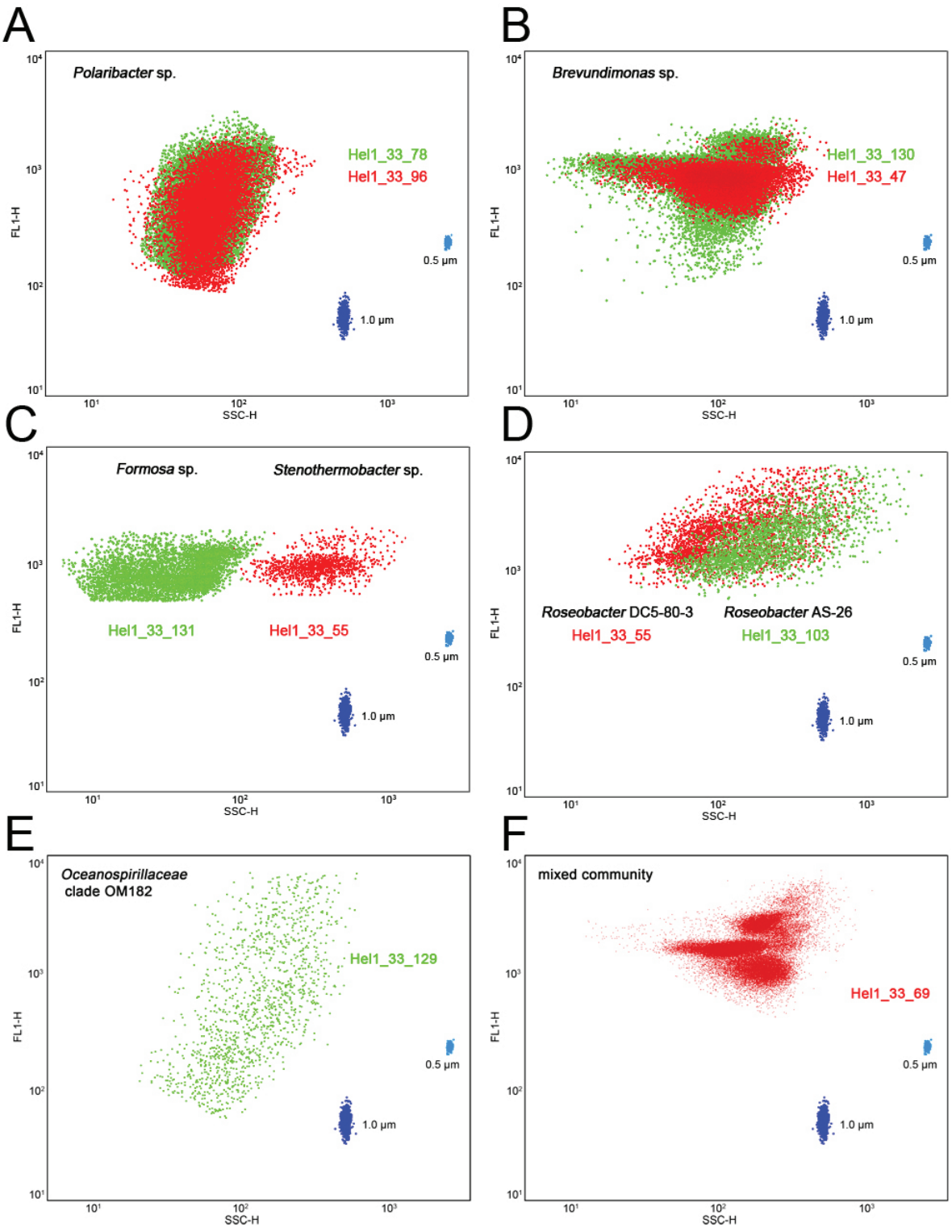
Figure 3.S2 Procedure for dilution cultivation of an untreated seawater sample. The seawater sample was diluted 1:10 with sterile artificial seawater (ASW), syringes, and Hungate tubes (black lid). Bacterial populations in portions of 1 nL, 10 nL and 100 nL, relative to the volume of seawater, were inoculated into polystyrene tubes (blue lid) filled with 10 mL ASW medium. As control, the medium was inoculated with the diluent ASW.

Influence of the HEPES buffer and agar on culturability

The artificial seawater medium HaHa had three differences to the marine agar HaHa. The solid medium HaHa was supplemented with 18 g/L washed agar. As buffer 50 mM HEPES were used for the marine agar HaHa and 2 mM bicarbonate for the artificial seawater HaHa. The carbon concentration of the marine agar HaHa was 2 g/L and of the artificial seawater HaHa 3 mg/L. We tested 10 pure liquid cultures for growth in the artificial seawater HaHa with a carbon concentration of 2 g/L. All 10 pure liquid cultures did grow under these elevated carbon concentration conditions. To test a putative negative effect of the agar on growth, the same pure cultures were inoculated into a medium composed of 2/3 artificial seawater with 18 g/L washed agar at pH 7.5 and 1/3 artificial seawater HaHa. All pure cultures did grow in the artificial seawater medium with washed bacto agar.

As a test for an influence of HEPES buffer on growth, the ASW medium was supplemented with either 2 mM bicarbonate buffer, 2 mM or 50 mM HEPES at pH 7.5. Three strains that were cultivated on agar plates and 10 pure cultures that grew in liquid medium only were incubated in the three media for 2 months. All bacteria from the agar plate were able to grow in all three media. In contrast, all strains from the liquid medium grew in the medium with 2 mM bicarbonate, weak or not in the 2 mM HEPES buffered medium, and not in the 50 mM HEPES medium.

Figure 3.S3 ***(facing page)*** Structure of dilution cultures of (A) *Polaribacter* sp. Hel1_33_78 and Hel1_33_96, (B) *Brevundimonas* sp. Hel1_33_47 and Hel1_33_130, (C) Hel1_33_131 and *Stenothermobacter* sp. Hel1_33_55, (D) *Roseobacter* clade DC5-80-3 strain Hel1_33_55 and clade AS-26 strain Hel1_33_103, and (E) OM182 strain Hel1_33_129. Bivariant dot plots of the two parameters of flow cytometry: side light scatter intensity (SSC-H, representing complexity of the cell) and SYBRGreen fluorescence intensity (FL1-H, reflecting DNA content). Beads with a diameter of 0.5 μm- (light blue) and 1.0 μm (dark blue) were used as an internal standard of fluorescence intensity. The dilution culture Hel1_33_69 was a mixture of different microorganisms confirmed by microscopy and 16S rRNA gene sequencing.


A
Polaribacter sp.
Hel1_33_78
Hel1_33_96
0.5 µm
1.0 µm
B
Brevundimonas sp.
Hel1_33_130
Hel1_33_47
0.5 µm
1.0 µm
C
Formosa sp.
Stenothermobacter sp.
Hel1_33_131
Hel1_33_55
0.5 µm
1.0 µm
D
Roseobacter DC5-80-3
Roseobacter AS-26
Hel1_33_55
Hel1_33_103
0.5 µm
1.0 µm
E
Oceanospirillaceae
clade OM182
Hel1_33_129
0.5 µm
1.0 µm
F
mixed community
Hel1_33_69
0.5 µm
1.0 µm
FL1-H
SSC-H

Proteorhodopsin analysis

The proteorhodopsin gene of *Flavobacteria* was amplified with both primer pairs PR-Flavo-F/R and PR-Flavo-2F/R as described previously (Yoshizawa et al., 2012). Proteorhodopsin sequences of isolates, the MIMAS metagenome dataset (Teeling et al., 2012), and closely related sequences from GenBank (Benson et al., 2010) were aligned in MAFFT (Katoh et al., 2002) as protein sequences. The phylogenetic tree was constructed with maximum likelihood using a 40% base frequency filter in ARB (Ludwig et al., 2004).

The proteorhodopsin gene of all three *Polaribacter* spp. cultures were successfully PCR amplified with both primer sets, PR-Fla-1F/R and PR-Fla-2F/R. The sequences had 99.9% identity to each other and fall into the proteorhodopsin cluster (suppl. Fig. 3.S4) with the cultures *Polaribacter* sp. SA4-10 and sp. SA4-47, isolated from the Sea ice of the Sea of Ochotsk, Hokkaido, Japan (Yoshizawa et al., 2012). Furthermore, these proteorhodopsin sequences were 100% identical to sequences of the metagenome from the bacterial community decomposing the spring phytoplankton bloom (Teeling et al., 2012). The proteorhodopsin sequence of strain Hel1_33_7 was PCR amplified with the primer pair PR-Fla-2F/R only. This proteorhodopsin sequence was 99.9% identical to the phylogenetically uncharacterized proteorhodopsin clones NA13_R15_12 and NA11_R15_8 of the North Atlantic Ocean (Sabehi et al., 2005). Thus, our strain Hel1_33_7 confirmed proteorhodopsin in surface seawater, and further showed the phylogenetic affiliation to a novel genus in the family *Flavobacteriaceae*, phylum *Bacteroidetes*. Furthermore, these proteorhodopsin sequences were 100% identical to sequences of the metagenome from the bacterial community decomposing the spring phytoplankton bloom in 2009 (Teeling et al., 2012).

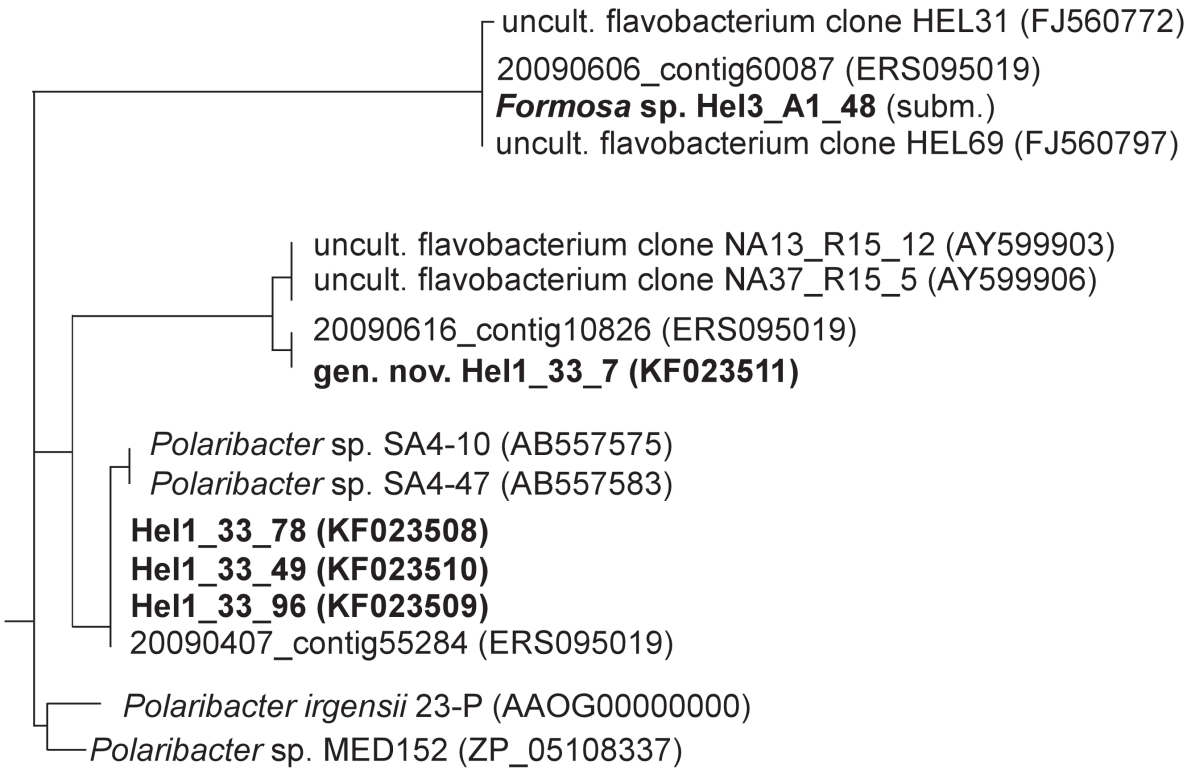


Figure 3.S4 Maximum likelihood tree of proteorhodopsin genes of isolates from spring 2010 (Hel1_33) and summer 2010 (Hel3_A1), from the metagenome of the spring phytoplankton bloom 2009 (20090606_contig) and other marine proteorhodopsin clones from the North Sea (HEL31, HEL69), the Yellow Sea (SA4), and the Central North Atlantic (NA).

HaHa medium

Artificial seawater medium: after Hahnke et al., 2014

1. Prepare and autoclave the modified Widdel flask

2. Prepare 1x ASW by dissolving the basal salts in autoclaved 1 L ultra pure water

	1 L 1x ASW
NaCl	26.37 g
$NaHCO_3$	0.19 g
$CaCl_2 \cdot 2\,H_2O$	1.47 g
KCl	0.72 g
KBr	0.10 g
H_3BO_3	0.02 g
$SrCl_2$	0.02 g

3. Add 0.5 mL NaF (0.006 g/mL, filtered)

4. ! Add magnetic stir bar !

5a. Fill up to 1 L with autoclaved ultra pure water
(5b. Check pH $<$ 7.0)

6. Autoclave and cool to 70 ℃

7. Add ultra pure water to a final volume of 1 L (autoclaved)

8. Cool to $<$ 40 ℃

9. Add sterile from the following stock solutions:

2.0 mL	Trace-element-solution	(autoclaved)
1.0 mL	Se-W-solution	(fresh autoclaved)
0.7 mL	KH_2PO_4-solution	(0.02 g/L, autoclaved)
1.0 mL	NH_4Cl-solution	(0.2 g/L, autoclaved)

10. Add carbon sources from the following stock solutions

0.6 mL	Glucose	(1 g/L, sterile filtered)
0.6 mL	Cellobioses	(1 g/L, sterile filtered)
0.6 mL	Yeast Extract (BioChemica)	(1 g/L, sterile filtered)
0.6 mL	Casaminoacids (Difco)	(1 g/L, sterile filtered)
0.6 mL	Tryptone Pepton (Difco)	(1 g/L, sterile filtered)

11. Add buffer from the following stock solutions (! pH $<$ 7.6!)

2.0 mL	$NaHCO_3$	(1 M, autoclaved, CO2 equilibrated)

12. Adjust pH to 7.5 with 1 M HCl or 1 M $NaHCO_3$ (autoclaved)

13. Add ultra pure water to a final volume of 1 L (autoclaved)

14. Filter the artificial seawater medium into sterile polystyrene tubes (15 mL, 188171, Greiner Bio-One, Frickenhausen, Germany) or Hungate tubes through a Sartolab P20 (0.2 μm poresize, 18075D, satorius, Göttingen, Germany) or P20 Plus (0.2 μm poresize, 18053D, satorius, Göttingen, Germany) filter

References

Alonso, C., Warnecke, F., Amann, R. and Pernthaler, J. (2007). High local and global diversity of *Flavobacteria* in marine plankton. *Environ Microbiol* **9**, 1253–1266.

Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R. and Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**, 1919–1925.

Azam, F. (1998). Microbial control of oceanic carbon flux: The plot thickens. *Science* **280**, 694–696.

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H., Knaust, F. et al. (2006). Whole genome analysis of the marine bacteroidetes '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**, 2201–2213.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Sayers, E. W. (2010). GenBank. *Nucleic Acids Res* **39**, D32–D37.

Bernardet, J.-F. (2010). *Bergey's Manual of Systematic Bacteriology. The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes Vol 4*, chapter Class II. *Flavobacteriia* class. nov. Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., Paster, B.J., Ward, N.L. et al. (eds), pp. 106–314. Springer, New York.

Bussmann, I., Philipp, B. and Schink, B. (2001). Factors influencing the cultivability of lake water bacteria. *J Microbiol Meth* **47**, 41–50.

Button, D. K., Schut, F., Quang, P., Martin, R. and Robertson, B. R. (1993). Viability and isolation of marine bacteria by dilution culture - theory, procedures, and initial results. *Appl Environ Microbiol* **59**, 881–891.

Campbell, B. J., Yu, L., Heidelberg, J. F. and Kirchman, D. L. (2011). Activity of abundant and rare bacteria in a coastal ocean. *Proc Natl Acad Sci USA* **108**, 12776–12781.

Carini, P., Steindler, L., Beszteri, S. and Giovannoni, S. J. (2012). Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. *ISME J* **7**, 592–602.

Cho, J. C. and Giovannoni, S. J. (2004). Cultivation and growth characteristics of a diverse group of oligotrophic marine *Gammaproteobacteria*. *Appl Environ Microbiol* **70**, 432–440.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M. et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. *Nucleic Acids Res* **37**, D141–D145.

Connon, S. A. and Giovannoni, S. J. (2002). High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol* **68**, 3878–3885.

Daims, H., Brühl, A., Amann, R., Schleifer, K.-H. and Wagner, M. (1999). The domain-specific probe EUB338 is insufficient for the detection of all *Bacteria*: Development and evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22**, 434–444.

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., Clardy, J. and Lewis, K. (2010). Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem Biol* **17**, 254–264.

Eilers, H., Pernthaler, J., Glöckner, F. O. and Amann, R. (2000). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66**, 3044–3051.

Eilers, H., Pernthaler, J., Peplies, J., Glöckner, F. O., Gerdts, G. and Amann, R. (2001). Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. *Appl Environ Microbiol* **67**, 5134–5142.

Fernández-Gómez, B., Richter, M., Schüler, M., Pinhassi, J., Acinas, S., González, J., J. M. and Pedrós-Alió, C. (2013). Ecology of marine Bacteroidetes: a comparative genomics approach. *ISME J* **7**, 1026–1037.

Fischer, B. (1894). *Die Bakterien des Meeres nach den Untersuchungen der Plankton-Expedition: unter gleichzeitiger Berücksichtigung einiger älterer und neuerer Untersuchungen.* Lipsius and Tischer, Kiel, Germany.

Frigaard, N. U., Martinez, A., Mincer, T. J. and DeLong, E. F. (2006). Proteorhodopsin lateral gene transfer between marine planktonic Bacteria and Archaea. *Nature* **439**, 847–850.

Fuchs, B. M., Zubkov, M. V., Sahm, K., Burkill, P. H. and Amann, R. (2000). Changes in community composition during dilution cultures of marine bacterioplankton as assessed by flow cytometric and molecular biological techniques. *Environ Microbiol* **2**, 191–201.

Giovannoni, S. J., Britschgi, T. B., Moyer, C. L. and Field, K. G. (1990). Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**, 60–63.

Giovannoni, S. J., Tripp, H. J., Givan, S., Podar, M., Vergin, K. L., Baptista, D., Bibbs, L., Eads, J., Richardson, T. H., Noordewier, M. et al. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**, 1242–1245.

Glöckner, F. O. and Joint, I. (2010). Marine microbial genomics in Europe: current status and perspectives. *Microb Biotechnol* **3**, 523–530.

Gobler, C. J., Norman, C., Panzeca, C., Taylor, G. T. and Sañudo-Wilhelmy, S. A. (2007). Effect of B-vitamins (B_1, B_{12}) and inorganic nutrients on algal bloom dynamics in a coastal ecosystem. *Aquat Microb Ecol* **49**, 181–194.

Gómez-Pereira, P. R., Fuchs, B. M., Alonso, C., Oliver, M. J., van Beusekom, J. E. E. and Amann, R. (2010). Distinct flavobacterial communities in contrasting water masses of the North Atlantic Ocean. *ISME J* **4**, 472–487.

Gómez-Pereira, P. R., Schüler, M., Fuchs, B. M., Bennke, C., Teeling, H., Waldmann, J., Richter, M., Barbe, V., Bataille, E., Glöckner, F. O. et al. (2012). Genomic content of uncultured *Bacteroidetes* from contrasting oceanic provinces in the North Atlantic Ocean. *Environ Microbiol* **14**, 52–66.

Hahnke, R. and Harder, J. (2013). Phylogenetic diversity of *Flavobacteria* isolated from the North Sea on solid media. *Syst Appl Microbiol* **in press**.

Hahnke, R. L., Bennke, C. M., Fuchs, B. M., Mann, A. J., Teeling, H., Amann, R. and Harder, J. (2013). Pure culture isolation of marine heterotrophic bacteria benefiting from a coastal diatom bloom. *Environ Microbiol* **in prep.**

Hall, P. and Aller, R. (1992). Rapid, small-volume, flow injection analysis for ΣCO_2 and NH_4^+ in marine and freshwaters. *Limnol Oceanogr* **37**, 1113–1119.

Hankeln, W., Wendel, N. J., Gerken, J., Waldmann, J., Buttigieg, P. L., Kostadinov, I., Kottmann, R., Yilmaz, P. and Glöckner, F. O. (2011). CDinFusion – submission-ready, on-line integration of sequence and contextual data. *PLoS One* **6**, e24797.

Hugenholtz, P. and Tyson, G. (2008). Microbiology: Metagenomics. *Nature* **455**, 481–483.

Ivanova, E. P., Alexeeva, Y. V., Flavier, S., Wright, J. P., Zhukova, N. V., Gorshkova, N. M., Mikhailov, V. V., Nicolau, D. V. and Christen, R. (2004). *Formosa algae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *Int J Syst Evol Microbiol* **54**, 705–711.

Joint, I., Mühling, M. and Querellou, J. (2010). Culturing marine bacteria – an essential prerequisite for biodiscovery. *Microb Biotechnol* **3**, 564–575.

Kaeberlein, T., Lewis, K. and Epstein, S. S. (2002). Isolating 'uncultivable' microorganisms in pure culture in a simulated natural environment. *Science* **296**, 1127–1129.

Kassabgy, M. (2011). *Diversity and abundance of Gammaproteobacteria during the winter-spring transition at station Kabeltonne (Helgoland).*

Ph.D. thesis, Department of Biology and Chemistry, University of Bremen, Bremen, Germany.

Katoh, K., Misawa, K., Kuma, K. and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059–3066.

Kirchman, D. L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O. (2012). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res* **41**, e1.

Konstantinidis, K. T. and DeLong, E. F. (2008). Genomic patterns of recombination, clonal divergence and environment in marine microbial populations. *ISME J* **2**, 1052–1065.

Konstantinidis, K. T. and Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proc Nat Acad Sci USA* **102**, 2567–2572.

Lennon, J. T. and Jones, S. E. (2011). Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat Rev Microbiol* **9**, 119–130.

Li, H., Yu, Y., Luo, W., Zeng, Y. and Chen, B. (2009). Bacterial diversity in surface sediments from the Pacific Arctic Ocean. *Extremophiles* **13**, 233–246.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G. et al.

(2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Malmstrom, R. R., Straza, T. R. A., Cottrell, M. T. and Kirchman, D. L. (2007). Diversity, abundance, and biomass production of bacterial groups in the western Arctic Ocean. *Aquat Microb Ecol* **47**, 45–55.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. and Schleifer, K. H. (1996). Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiology* **142**, 1097–1106.

Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**, 593–600.

Martens, E. C., Lowe, E. C., Chiang, H., Pudlo, N. A., Wu, M., McNulty, N. P., Abbott, D. W., Henrissat, B., Gilbert, H. J., Bolam, D. N. et al. (2011). Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol* **9**, e1001221.

Massana, R. and Jürgens, K. (2003). Composition and population dynamics of planktonic bacteria and bacterivorous flagellates in seawater chemostat cultures. *Aquat Microb Ecol* **32**, 11–22.

Morris, J. J., Lenski, R. E. and Zinser, E. R. (2012). The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *mBio* **3**, e00036–12.

Mulligan, C., Fischer, M. and Thomas, G. H. (2011). Tripartite ATP-independent periplasmic (TRAP) transporters in bacteria and archaea. *FEMS Microbiol Rev* **35**, 68–86.

Muyzer, G., Teske, A., Wirsen, C. and Jannasch, H. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**, 165–172.

Nedashkovskaya, O. I., Kim, S. B., Vancanneyt, M., Snauwaert, C., Lysenko, A. M., Rohde, M., Frolova, G. M., Zhukova, N. V., Mikhailov, V. V., Bae, K. S. et al. (2006). *Formosa agariphila* sp. nov., a budding bacterium of the family *Flavobacteriaceae* isolated from marine environments, and emended description of the genus *Formosa*. *Int J Syst Evol Microbiol* **56**, 161–167.

Neef, A. (1997). *Application of in situ identification of bacteria to population analysis in complex microbial communities*. Ph.D. thesis, Department of Microbiology, Technical University of Munich, Munich, Germany.

Neef, A., Amann, R., Schlesner, H. and Schleifer, K.-H. (1998). Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. *Microbiology* **144**, 3257–3266.

Ning, Z. M., Cox, A. J. and Mullikin, J. C. (2001). SSAHA: a fast search method for large DNA databases. *Genome Res* **11**, 1725–1729.

Nossa, C. W., Oberdorf, W. E., Yang, L. Y., Aas, J. A., Paster, B. J., DeSantis, T. Z., Brodie, E. L., Malamud, D., Poles, M. A. and Pei, Z. H. (2010). Design of 16S rRNA gene primers for 454 pyrosequencing of the human foregut microbiome. *World J Gastroenterol* **16**, 4135–4144.

Ouverney, C. C. and Fuhrman, J. A. (1999). Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. *Appl Environ Microbiol* **65**, 1746–1752.

Overmann, J. (2010). *Geomicrobiology: molecular and environmental perspective*, chapter Novel cultivation strategies for environmentally important microorganisms. Barton LL, Mandl M, Loy A (eds), pp. 69–89. Springer Science and Business Media B.V., Springer, Berlin, Germany.

Pedrós-Alió, C. (2006). Marine microbial diversity: can it be determined? *Trends Microbiol* **14**, 257–263.

Pfennig, N., and Trüper, H. G. (1981). *The Prokaryotes*, chapter Isolation of members of the families *Chromatiaceae* and *Chlorobiaceae*. Starr, M.P. and Stolp, H. and Trüper, H. G. and Balows, A. and Schlegel, H. G. (eds), pp. 279–289. Springer, Berlin, Germany.

Pham, V. D., Konstantinidis, K. T., Palden, T. and DeLong, E. F. (2008). Phylogenetic analyses of ribosomal DNA-containing bacterioplankton genome fragments from a 4000 m vertical profile in the North Pacific Subtropical Gyre. *Environ Microbiol* **10**, 2313–2330.

Pinhassi, J., Pujalte, M. J., Macian, M. C., Lekunberri, I., Gonzalez, J. M., Pedros-Alio, C. and Arahal, D. R. (2007). *Reinekea blandensis* sp. nov., a marine, genome-sequenced gammaproteobacterium. *Int J Syst Evol Microbiol* **57**, 2370–2375.

Pinhassi, J., Zweifel, U. L. and Hagström, Å. (1997). Dominant marine bacterioplankton species found among colony-forming bacteria. *Appl Environ Microbiol* **63**, 3359–3366.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W. G., Peplies, J. and Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**, 7188–7196.

Radach, G., Berg, J. and Hagmeier, E. (1990). Long-term changes of the annual cycles of meteorological, hydrographic, nutrient and phytoplankton time series at Helgoland and at LV ELBE 1 in the German Bight. *Continent Shelf Res* **10**, 305–328.

Rappé, M. S., Connon, S. A., Vergin, K. L. and Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630–633.

Reichenbach, H. (1981). Taxomony of the gliding bacteria. *Annu Rev Microbiol* **35**, 339–364.

Riedel, T., Tomasch, J., Buchholz, I., Jacobs, J., Kollenberg, M., Gerdts, G., Wichels, A., Brinkhoff, T., Cypionka, H. and Wagner-Döbler, I. (2010). Constitutive expression of the proteorhodopsin gene by a flavobacterium strain representative of the proteorhodopsin-producing microbial community in the North Sea. *Appl Environ Microbiol* **76**, 3187–3197.

Romanenko, L. A., Schumann, P., Rohde, M., Mikhailov, V. V. and Stackebrandt, E. (2004). *Reinekea marinisedimentorum* gen. nov., sp. nov., a novel gammaproteobacterium from marine coastal sediments. *Int J Syst Evol Microbiol* **54**, 669–673.

Rosselló-Mora, R. and Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Sabehi, G., Loy, A., Jung, K. H., Partha, R., Spudich, J. L., Isaacson, T., Hirschberg, J., Wagner, M. and Béjà, O. (2005). New insights into metabolic properties of marine bacteria encoding proteorhodopsins. *PLoS Biol* **3**, 1409–1417.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sañudo-Wilhelmy, S. A., Cutter, L. S., Durazo, R., Smail, E. A., Gómez-Consarnau, L., Webb, E. A., Prokopenko, M. G., Berelson, W. M. and Karl, D. M. (2012). Multiple B-vitamin depletion in large areas of the coastal ocean. *Proc Natl Acad Sci USA* **109**, 14041–14045.

Sapp, M., Schwaderer, A., Wiltshire, K., Hoppe, H.-G., Gerdts, G. and Wichels, A. (2007a). Species-specific bacterial communities in the phycosphere of microalgae? *Microb Ecol* **53**, 683–699.

Sapp, M., Wichels, A., Wiltshire, K. H. and Gerdts, G. (2007b). Bacterial community dynamics during the winter-spring transition in the North Sea. *FEMS Microbiol Ecol* **59**, 622–637.

Schauer, K., Rodionov, D. A. and de Reuse, H. (2008). New substrates for TonB-dependent transport: do we only see the 'tip of the iceberg'? *Trends Biochem Sci* **33**, 330–338.

Schloss, P. D. and Handelsman, J. (2004). Status of the microbial census. *Microbiol Mol Biol Rev* **68**, 686–691.

Schneider, E., Eckey, V., Weidlich, D., Wiesemann, N., Vahedi-Faridi, A., Thaben, P. and Saenger, W. (2012). Receptor-transporter interactions of canonical ATP-binding cassette import systems in prokaryotes. *Eur J Cell Biol* **91**, 311–317.

Sharma, A. K., Spudich, J. L. and Doolittle, W. F. (2006). Microbial rhodopsins: functional versatility and genetic mobility. *Trends Microbiol* **14**, 463–469.

Stackebrandt, E. and Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today* **33**, 152–155.

Staley, J. T. and Konopka, A. (1985). Measurements of in situ activities of nonphotosynthetic mikroorganisms in aquatic and terreestrial habitats. *Annu Rev Microbio* **39**, 321–346.

Stevens, H., Simon, M. and Brinkhoff, T. (2009). Cultivable bacteria from bulk water, aggregates, and surface sediments of a tidal flat ecosystem. *Ocean Dynam* **59**, 291–304.

Stingl, U., Desiderio, R. A., Cho, J.-C., Vergin, K. L. and Giovannoni, S. J. (2007). The SAR92 clade: an abundant coastal clade of culturable marine bacteria possessing proteorhodopsin. *Appl Environ Microbiol* **73**, 2290–2296.

Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J. et al. (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336**, 608–611.

Valentin, R. C., Shapiro, B. M. and Stadtman, E. R. (1968). Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry* **7**, 2143–2152.

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D. Y., Paulsen, I., Nelson, K. E.,

Nelson, W. et al. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**, 66–74.

Wallner, G., Amann, R. and Beisker, W. (1993). Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14**, 136–143.

Widdel, F. and Bak, F. (1992). *The Prokaryotes Vol 2*, chapter Gram-negative mesophilic sulfate-reducing bacteria. Balows A., Trüper H.G., Dworkin M. and Harder W. (eds), pp. 3352–3378. Springer, Berlin, Germany.

Winkelmann, N. and Harder, J. (2009). An improved isolation method for attached-living *Planctomycetes* of the genus *Rhodopirellula*. *J Microbiol Meth* **77**, 276–284.

Yan, S., Fuchs, B. M., Lenk, S., Harder, J., Wulf, J., Jiao, N.-Z. and Amann, R. (2009). Biogeography and phylogeny of the NOR5/OM60 clade of *Gammaproteobacteria*. *Syst Appl Microbiol* **32**, 124–139.

Yarza, P., Ludwig, W., Euzéby, J., Amann, R., Schleifer, K.-H., Glöckner, F. O. and Rosselló-Móra, R. (2010). Update of the All-Species Living Tree Project based on 16S and 23S rRNA sequence analyses. *Syst Appl Microbiol* **33**, 291–299.

Yooseph, S., Sutton, G., Rusch, D. B., Halpern, A. L., Williamson, S. J., Remington, K., Eisen, J. A., Heidelberg, K. B., Manning, G., Li, W. Z. et al. (2007). The Sorcerer II Global Ocean Sampling expedition: expanding the universe of protein families. *PLoS Biol* **5**, 432–466.

Yoshizawa, S., Kawanabe, A., Ito, H., Kandori, H. and Kogure, K. (2012). Diversity and functional analysis of proteorhodopsin in marine *Flavobacteria*. *Environ Microbiol* **14**, 1240–1248.

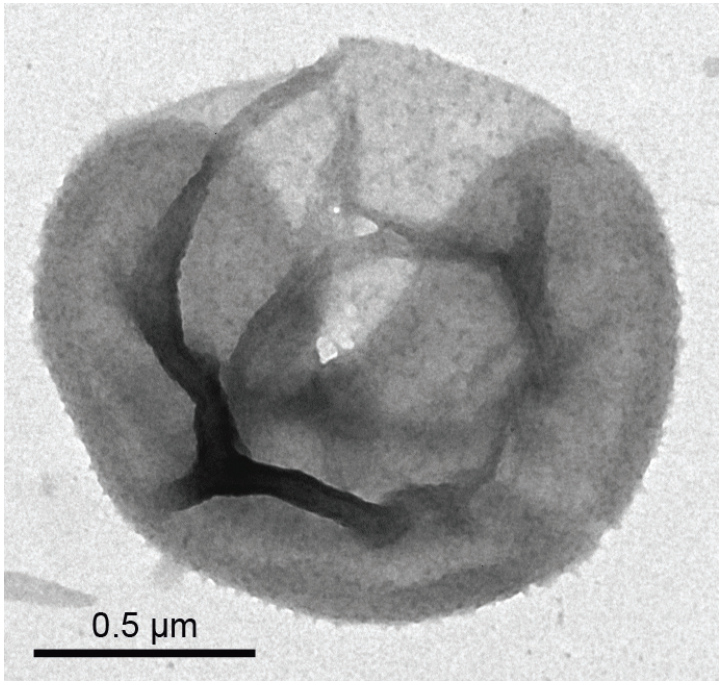
Yu, Z. T. and Mohn, W. W. (2001). Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic spacer analyses and 16S ribosomal DNA sequencing. *Appl Environ Microbiol* **67**, 1565–1574.

Zengler, K. (2009). Central role of the cell in microbial ecology. *Microbiol Mol Biol Rev* **73**, 712–729.

ZoBell, C. E. (1946). *Marine microbiology. A monograph of hydrobacteriology.* Chronica Botanica Co., Waltham, Mass. U.S.A.

Zubkov, M. V. and Burkill, P. H. (2006). Syringe pumped high speed flow cytometry of oceanic phytoplankton. *Cytometry Part A* **69A**, 1010–1019.

Zubkov, M. V., Fuchs, B. M., Burkill, P. H. and Amann, R. (2001). Comparison of cellular and biomass specific activities of dominant bacterioplankton groups in stratified waters of the Celtic Sea. *Appl Environ Microbiol* **67**, 5210–5218.



'*Polaribacter forsetii*' surrounded by a glycoconjugate matrix.

Chapter 4

Carbohydrate utilization and initial taxonomic description of North Sea isolates

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Contributions to the manuscript:

R.L.H. and J.H. designed research and project outline. R.L.H. performed physiological studies. R.L.H. conceived and wrote the manuscript. J.H. revised the manuscript.

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4.1 Abstract

During phytoplankton bloom senescence distinct bacterioplankton populations serially succeeded. Based on *in situ* expressed transporters and glycoside hydrolases it was suggested that algae derived carbohydrates provided a series of ecological niches. We studied the physiological characteristics and carbohydrate substrate specificity of strains which were isolated as representatives of phytoplankton decomposing populations. Glycoconjugate fibers on *Polaribacter* cell surface mediated aggregate formation and strong attachment. *Formosa* strains were linked to each other by strings of pearl-like structures in a three dimensional network that caused an increasing medium viscosity. *Reinekea* sp. grew on all tested mono-, di-, trisaccharides, but not on tested polysaccharides. *Formosa* strains were able to grow on un-substituted polysaccharides, whereas *Polaribacter* strains additionally grew on substituted polysaccharides. These physiological traits provided further evidence that *Formosa*, *Polaribacter*, and *Reinekea* species could prevail in different ecological niches during algae decay. On the basis of 16S rRNA sequence analysis and preliminary phenotypic analysis novel species were proposed. '*Formosa flavarachnoidea*' (Hel3_A1_48) and '*Formosa forsetii*' (Hel1_33_131) belonged to the genus *Formosa* (*Flavobacteria*). '*Polaribacter forsetii*' (Hel1_33_49), '*Polaribacter frigidimaris*' (Hel1_33_78), and '*Polaribacter adhaesivus*' (Hel1_33_96) belonged to the genus *Polaribacter* (*Flavobacteria*). '*Reinekea forsetii*' (Hel1_31_D35) belonged to the genus *Reinekea* (*Gammaproteobacteria*).

4.2 Introduction

Heterotrophic bacteria play an important role in cycling of organic matter in the ocean by remineralizing more than 50% of the net primary production (Azam, 1998). During phytoplankton bloom senescence, bacterial cell numbers, growth rates and hydrolytic enzyme activity increased significantly (Smith et al., 1995). In experimentally induced as well as naturally occurring phytoplankton blooms the responding bacterioplankton community consisted mainly of *Bacteroidetes*, *Alphaproteobacteria* and *Gammaproteobacteria* (Pinhassi et al., 2004; Riemann et al., 2000; Schäfer et al., 2001; Tada et al., 2011). *Flavobacteria* were shown to dominate the bacterioplankton that consumed proteins, N-acetylglucosamine and polysaccharides (e.g. chitin), whereas *Alphaproteobacteria* and *Gammaproteobacteria* dominated the amino acids consuming part of the bacterioplankton, revealed by microautoradiography of estuarine and coastal bacterioplankton (Cottrell and Kirchman, 2000).

On the genus level, the peaking of distinct bacterial populations characterized the bacterioplankton community after the spring phytoplankton bloom in shallow coastal waters of the North Sea (Teeling et al., 2012). In the early phase of algae decay, *Ulvibacter*, *Formosa* (*Flavobacteriaceae*) and the *Roseobacter* NAC11-7 lineage (*Alphaproteobacteria*) succeeded, whereas *Polaribacter* (*Flavobacteriaceae*), the *Roseobacter* clade-affiliated (RCA) lineage (*Alphaproteobacteria*), SAR92 and *Reinekea* (*Gammaproteobacteria*) spiked in the late phase (Teeling et al., 2012). It was proposed that the ecological niche of *Reinekea* was an uptake of peptides and monosaccharides, based on the observed expression of mainly ABC type and TRAP transporters. Mono- and oligosaccharides are the result of polysaccharides degradation by extracellular glycoside hydrolases. Based on expressed glycoside hydrolases and sulfatases, it was suggested that the early blooming

Formosa potentially decomposed non-substituted laminarin, whereas the late blooming *Polaribacter* were able to decompose sulfated polysaccharides (Teeling et al., 2012).

Representatives of the three genera *Formosa*, *Polaribacter* and *Reinekea* were isolated from the seawater of Helgoland in 2010, and showed high similarities to 16S rRNA and functional gene sequences of the bacterioplankton metagenome in spring 2009 (Hahnke et al., 2014). We studied metabolic potentials and nutrient requirements of these marine *Flavobacteriaceae* and *Gammaproteobacteria* isolates to gain further insights into ecological roles of bacterioplankton populations in the remineralization of algae derived carbohydrates. The focus was on the utilization of mono-, di-, tri- and polysaccharides by strains Hel3_A1_48, Hel1_33_131, Hel1_33_49, Hel1_33_78, Hel1_33_96 and Hel1_31_D35. Furthermore, the results of this study should provide target strains and polysaccharides to initialize proteomic studies on potential polysaccharide utilization loci.

4.3 Material and methods

Artificial seawater (ASW) and all media were prepared with sterile filtered (0.2 μm polycarbonate filter) ultra pure water (Aquintus system, membraPure, Germany) with a electrical resistivity of 18.3 MΩ m.

ASW was prepared following the recipe of Widdel and Bak (1992) as described by Winkelmann and Harder (2009). The marine media HaHa, HaHa_100 and HaHa_100V were prepared as described by Hahnke and colleagues (2014). The HaHa_min medium was identical to the HaHa medium, with an addition of 1.1 mL/L NH_4Cl (5.0 g/L, autoclaved) and 0.1 mL/L KH_2PO_4 (50 g/L, autoclaved) providing 100 μM carbon, 103 μM ammonium and 16 μM phosphate. The HaHa_minV medium was identical to the HaHa_min medium with the addition of 1 mL/L 7-vitamin solution (Winkel-

mann and Harder, 2009), 1 mL/L vitamin B_{12} solution (Widdel and Bak, 1992), 1 mL/L thiamine solution (Winkelmann and Harder, 2009), and 1 mL/L riboflavin solution (Winkelmann and Harder, 2009). All media were buffered with 2 mM $NaHCO_3$ (Widdel and Bak, 1992) at pH 7.5. Evaporated water was replaced with autoclaved ultra pure water.

Mono-, di- and trisaccharides

The monosaccharides D-galactose, D-mannose, L-rhamnose, D-fructose, D-mannitol, D-glucose, DL-xylose, L-arabinose and D-arabinose, the disaccharides trehalose, D-sucrose, D-maltose and D-cellobiose, the trisaccharides raffinose and N-acetyl-D-glucosamine were dissolved in ultra pure water (10 g/L). The substrate solutions were adjusted to pH 7 with 1 M NaOH or HCl and sterile filtered through a 0.2 μm pore size filter (0.2 μm filter, Minisart, Sartorius, Göttingen, Germany). The HaHa_min and HaHa_minV media were supplemented with 100 μL of the substrate solutions per 10 mL medium (0.1 g/L final concentration).

Polysaccharides

For cellulose preparation (structural affected), Whatman filter paper (Grade 595 1/2, Whatman™, GE Healthcare, Freiburg, Germany) was cut into pieces and washed three times with ultra pure water followed by 70% ethanol. Filter paper pieces were autoclaved in ultra pure water at 121 ℃for 21 min. Agar, xylan, κ- and ι-carrageenan were prepared following the protocol of Widdel and Bak (1992). The double concentrated ASW was autoclaved and mixed with 4% (w/v) agar (Bacto™, BD Bionutrients™, BD Biosciences, Sparks, MD, USA), xylan (4414.1, Carl Roth, Karlsruhe, Germany), κ-carrageenan (22048, Sigma-Aldrich, Hamburg, Germany) or ι-carrageenan (22045, Sigma-Aldrich, Hamburg, Germany) were autoclaved in different bottles at 121 ℃ for 21 min. The double concentrated ASW and

one of the polymeric substances were mixed 1:1 (v/v) by stirring at 80 °C. The mixture was adjusted to pH 7 and poured into polypropylene Petri dish. Colloidal chitin was prepared as described by Souza (2009). 10 g of chitin (powdered crab shells, C-9213, Sigma-Aldrich, Hamburg, Germany) were added to 150 ml 37% HCl and stirred at room temperature for 5 hours. The dissolved chitin was filtered through glass wool. Under vigorous stirring, the addition of 400 mL 50% ethanol (final concentration) caused precipitation of the chitin. The chitin suspension was filtered on glass-fiber filter (alternatively top-bottle filter can be used for faster filtration) and washed with autoclaved ultra pure water until pH 7. The colloidal chitin was washed with 70% ethanol and air dried. Laminarin from *Laminaria saccharina* (L-1760, Sigma-Aldrich, Hamburg, Germany) was washed three times with ultra pure water and pasteurized three times by incubation in ultra pure water at 70 °C for 1 h and washing with autoclaved ultra pure water. An intensive treatment with ultra pure water was important to exclude D-mannitol impurities. Glycogen (G-8751, Sigma-Aldrich, Hamburg, Germany) was dissolved in autoclaved artificial seawater and sterilized through a 0.2 μm sterile filter (Minisart, Sartorius, Göttingen, Germany). Gelatin powder (4582.3, Carl Roth, Karlsruhe, Germany) was dissolved in 70 °C autoclaved artificial seawater and poured into polypropylene Petri dishes.

A portion of the prepared sterile polysaccharides was placed into a polystyrene tube which was filled up to a volume of 10 mL with either HaHa_min or HaHa_minV medium. A portion of the prepared polysaccharides was incubated in HaHa_100V medium at room temperature for at least one weak to test for contaminating bacteria.

Cultivation

Substrate test were performed in duplicates and repeated once. 100 μL of culture were inoculated into 10 mL HaHa_min or HaHa_minV medium supplemented with mono-, di-, tri- or polysaccharides. To test for growth inhibition by the prepared saccharides and polysaccharides the strains were inoculated into HaHa_100 or HaHa_100V medium. Growth was detected by biomass formation in duplicates, against the strain in HaHa_min or HaHa_minV medium, and the saccharide in HaHa_100V medium.

Cell staining

For visualization of cells that are attached to the polysaccharides cellulose or laminarin, small pieces of the polysaccharides were incubated with 4´6-diamidino-2-phenylindole (DAPI, 1 μg/ml ASW) at room temperature for 10 min. Excess DAPI and salts were thoroughly removed by washing the polysaccharide with sterile ultra pure water and 70% ethanol. The polysaccharide piece was mounted on glass slides with Citifluor and VectaShield (4:1) and DAPI stained cells were determined on a Zeiss Axioplan II Imaging epifluorescence microscope.

Transmission electron microscopy

For negative staining, samples were absorbed onto carbon film, washed in TE buffer (20 mM Tris/HCl, 1 mM EDTA, pH 6.9), stained with 4% (w/v) aqueous uranyl acetate (pH 4.5) according to the method of Valentine et al. (1968) and picked up with 300-mesh copper grids. After air-drying, samples were examined in a EM109 transmission electron microscope (TEM) at an acceleration voltage of 80 kV and at calibrated magnifications.

4.4 Results and discussion

General characteristics of the strains

All of the *Flavobacteriaceae* strains investigated had either yellow or orange pigments. Flexirubin pigments were not detected. All strains studied, except '*Formosa flavarachnoidea*', were cold-adapted, with growth occurring at 0 ℃ to 23 ℃ or 26 ℃ in marine HaHa_100 medium. Optimal growth yields occurred at 12 ℃ to 16 ℃. Strain '*Formosa flavarachnoidea*' grew between 8 ℃ and 25 ℃, with optimal growth at 22 ℃. None of the strains studied grew at 30 ℃ or higher in HaHa_100V medium. The original seawater of Helgoland had a temperature of 7.4 ℃ at the time of sampling and usually ranges between 0 ℃ and 20 ℃ (Gerdts et al., 2004). All studied strains shared the utilization of amino acids and oligopeptides from casamino acids, tryptone peptone, yeast extract, and gelatin. For '*Polaribacter frigidimaris*' the hydrolysis of gelatin was not observed, but growth occurred. Vitamins were not required for growth, except for strains '*Formosa flavarachnoidea*' and '*Reinekea forsetii*'. Common and differential phenotypic characteristics of the strains are listed in Tab. 4.1 and summarized below in the preliminary descriptions.

It was repeatedly hypothesized that the ecological role of *Bacteroidetes* is the decomposition of high molecular weight (HMW) organic matter (Ivanova et al., 2004), the initial step for the remineralization of organic matter in the ocean (Arnosti, 2010). The ability of the *Flavobacteriaceae* strains to attach and grow on a variety of polysaccharides (e.g. laminarin, cellulose, agar, κ-carrageenan) in contrast to *Reinekea* supports this role. '*Polaribacter forsetii*' (*Polaribacter* sp. Hel1_33_49) formed a pellet in liquid culture in contrast to the formation of soft cloudy aggregates of '*Polaribacter frigidimaris*' (*Polaribacter* sp. Hel1_33_78) and '*Polaribacter adhaesivus*' (*Polaribacter* sp. Hel1_33_96) (Fig. 4.2 and

Fig. 4.3). Net-like extracellular glycoconjugate fibers occupied the whole cell surface and were the basis for aggregate formation and strong attachment of '*Polaribacter frigidimaris*' and '*Polaribacter adhaesivus*'. Colonies of these strains were still attached to surfaces of different materials (e.g. polystyrene, cellulose, chitin) after centrifugation for 1 h at 2500x *g*. For strains '*Formosa flavarachnoidea*' (*Formosa* sp. Hel3_A1_48) and '*Formosa forsetii*' (*Formosa* sp. Hel1_33_131), appendages were observed that emanate from the cell surface with globules of variable size and distances (Fig. 4.1). Appendages connected the cells with each other, mediated aggregate formation, and resulted in an increased viscosity of the medium. Such properties were described from *Lentisphaera araneosa*, as a strategy to trap particles in the seawater induced by cobweb like structures (Cho et al., 2004). Vortexing destroyed *Formosa* cells, but the more robust globular structures appeared without alterations. Consequently, for further cultivation the *Formosa* strains were homogenized by gently inverting.

Furthermore, it was proposed that dedicated *Flavobacteria* are specialized for different fractions of complex organic matter, based on the extent of polysaccharide utilization loci (PUL) within a genome and their difference in composition between individual genomes of *Flavobacteria* (Bauer et al., 2006; Gómez-Pereira et al., 2012) and between distinct *Flavobacteria* populations during phytoplankton decomposition (Teeling et al., 2012). While *Formosa agariphila* DSM 15362^{T} was able to grow on all tested mono-, di-, trisaccharides, and N-acetyl-D-glucosamine, strain '*Formosa flavarachnoidea*' oxidized D-galactose and strain '*Formosa forsetii*' oxidized D-glucose and D-cellobiose exclusively, under the given cultivation conditions. All three *Formosa* strains were able to grow on laminarin (Fig. 4.2) and slightly (less biomass) on κ-carrageenan, but not on agar. '*Formosa flavarachnoidea*' grew on cellulose (structural affected) (Fig. 4.3) and carboxymethyl-cellulose, in contrast to *Formosa agariphila*

DSM 15362^{T} and '*Formosa forsetii*'. Strains *Polaribacter* sp. Hel1_85 and '*Polaribacter forsetii*' differed remarkably from strains '*Polaribacter frigidimaris*' and '*Polaribacter adhaesivus*', based on their spectrum of oxidized carbohydrates. Both *Polaribacter* strains '*Polaribacter frigidimaris*' and '*Polaribacter adhaesivus*' grew on all tested mono-, di-, trisaccharides and N-acetyl-D-glucosamine. The difference between strains '*Polaribacter frigidimaris*' and '*Polaribacter adhaesivus*' was the inability to grow on glycogen and the narrow temperature range of growth of strain '*Polaribacter frigidimaris*'. *Polaribacter* sp. Hel1_85 grew on D-galactose, D-mannose, D-mannitol, DL-xylose, DL-arabinose, D-sucrose, D-maltose and L-raffinose, but not on L-rhamnose, D-fructose, D-glucose, D-trehalose, D-cellobiose and N-acetyl-D-glucosamine. '*Polaribacter forsetii*' had a narrow carbohydrate spectrum and grew only on D-mannose, D-glucose, D-maltose and D-cellobiose.

Teeling and Fuchs et al. (Teeling et al., 2012) hypothesized different ecological niches of *Polaribacter* and *Formosa*, based on the potential decomposition of the more complex substituted polysaccharides by *Polaribacter* populations. This was reflected in the decomposition of un-substituted polysaccharides (e.g. laminarin, cellulose) by studied strains of the both genera *Formosa* and *Polaribacter*, in contrast to the decomposition of substituted polysaccharides (e.g. agar, xanthan, carrageenan) by all studied *Polaribacter* strains only.

As a result of polysaccharides degradation by extracellular glycoside hydrolases, monosaccharides and oligosaccharides become available that can be taken up by fast growing opportunistic bacteria of broad substrate spectra. Comparable to '*Reinekea forsetii*', *Reinekea blandensis* MED297^{T} was described with a broad substrate range of mono- and disaccharides (Choi and Cho, 2010; Pinhassi et al., 2007). '*Reinekea forsetii*' grew on all tested mono-, di-, trisaccharides, N-acetyl-D-glucosamine and N-acetylneuraminic

acid, slightly on xanthan and glycogen, but not on all other tested polysaccharides. While the oxidation of N-acetyl-D-glucosamine was described for *Reinekea blandensis* MED297^{T} and *Reinekea aestuarii* IMCC4489^{T}, none of the type strains had been tested for growth on N-acetylneuraminic acid (Pinhassi et al., 2007). Nevertheless, the finding of the NanAKE and NagAB cluster and the N-acetylneuraminic acid specific TRAP transporter in the genome of *Reinekea blandensis* MED297^{T} corroborates the utilization of N-acetylneuraminic acid by *Reinekea blandensis* MED297^{T} (Pinhassi et al., 2007; Vimr et al., 2004). This nutritional strategy is reflected by the *in situ* expression of mainly ABC type and TRAP transporters for the potential uptake of peptides, monosaccharides and other monomers, in the late phase of the decomposition of the spring phytoplankton (Teeling et al., 2012).

Description of '*Formosa flavarachnoidea*'

'*Formosa flavarachnoidea*' (flav.a.rach.no.i.de'a. L. adj. *flavus*, yellow; N.L. fem. adj. *arachnoidea*, similar to cobwebs; *flavarachnoidea*, pertaining to the yellow color and the cobweb like structures produced by the strain)

Cells were rod-shaped, 0.6 to 1.2 μm long and 0.5 to 0.7 μm wide. Non-motile. Cells occurred singly or as aggregates. Appendages composed of 50 nm globules and 10 μm to 100 μm long emanated from the cell surface. Appendages connected the cells with each other and formed a three dimensional network. Cells were destroyed by vortexing. Yellow-orange cell pellets were formed in liquid culture. Growth occurred from 8 ℃ to 25 ℃, with an optimum at 22 ℃. Divided by binary fission. Did not grow on marine agar HaHa and marine agar 2216. With marine HaHa_minV medium, growth occurred on casamino acids, peptone tryptone, yeast extract, gelatin, D-galactose, cellulose (structural affected filter paper), carboxymethyl cellulose and laminarin, weak growth on glycogen, xanthan and κ-carrageenan, but not on L-arabinose, D-arabinose, D-cellobiose, D-fructose, D-glucose, N-acetyl-D-glucosamine, D-maltose, D-mannitol, D-mannose, L-raffinose, L-rhamnose, D-sucrose, D-trehalose, DL-xylose, agar and ι-carrageenan. Vitamins were required.

The strain, Hel3_A1_48, was isolated from the seawater of Helgoland Roads, German Bight of the North Sea, Germany (54°11' N, 7°54' E).

Description of '*Formosa forsetii*'

'*Formosa forsetii*' (for.set'ti.i. N.L. gen. fem. n. *forsetii*, of Forseti, a god in Scandinavian mythology that lived on Helgoland, the German island from where the bacterium was isolated).

Cells were rod-shaped, 0.6 to 0.8 μm long and 0.5 to 0.6 μm wide. Non-motile. Cells occurred singly or as aggregates. Appendages emanated from the cell surface with globules of 50 to 80 nm size and varying distances. Appendages connected the cells with each other and formed a three dimensional network. Cells were destroyed by vortexing. Yellow-orange cell pellets were formed in liquid culture. Growth occurred from 4 ℃ to 19 ℃, with an optimum at 15 ℃. Divided by binary fission. Did not grow on marine agar HaHa and marine agar 2216. With marine HaHa_-min medium, growth occurred on casamino acids, peptone tryptone, yeast extract, gelatin, D-glucose, D-cellobiose and laminarin, weak growth on ι-carrageenan and κ-carrageenan, but not on D-arabinose, L-arabinose, D-fructose, D-galactose, N-acetyl-D-glucosamine, D-maltose, D-mannitol, D-mannose, L-raffinose, L-rhamnose, D-trehalose, D-sucrose, DL-xylose, cellulose (structural affected filter paper), carboxymethyl cellulose, agar, xanthan and glycogen. Vitamins were not required.

The strain, Hel1_33_131, was isolated from the seawater of Helgoland Roads, German Bight of the North Sea, Germany (54°11' N, 7°54' E).

Description of '*Polaribacter forsetii*'

'*Polaribacter forsetii*' (for.set'ti.i. N.L. gen. masc. n. *forsetii*, of Forseti, a god in Scandinavian mythology that lived on Helgoland, the German island from where the bacterium was isolated).

Cells were rod-shaped, 0.5 to 2.0 μm long and 0.5 to 0.8 μm wide. Non-motile. Cells occurred singly or as aggregates of 2 to 3 cells. Appendages at the cell surface were observed with a diameter of less than 50 nm and a length of more than 10 μm, up to 100 μm. Yellow-orange cell pellets were formed in liquid culture. Growth occurred between 2 ℃and 22 ℃, with an optimum at 14 ℃. Divided by binary fission. Did not grow on marine agar HaHa and marine agar 2216. With marine HaHa_min medium, growth occurred on casamino acids, peptone tryptone, yeast extract, gelatin, L-arabinose, D-arabinose, D-cellobiose, D-glucose, D-maltose, D-mannose, L-raffinose, glycogen, cellulose (structural affected filter paper), carboxymethyl cellulose, laminarin, agar, xanthan, κ-carrageenan and ι-carrageenan, but not on D-fructose, D-galactose, N-acetylD-glucosamine, D-mannitol, L-rhamnose, D-sucrose, D-trehalose and DL-xylose. Vitamins were not required.

The strain, Hel1_33_49, was isolated from the seawater of Helgoland Roads, German Bight of the North Sea, Germany (54°11' N, 7°54' E).

Description of '*Polaribacter frigidimaris*'

'*Polaribacter frigidimaris*' (fri.gid.i.ma.r'is. L. masc. adj. *frigidus*, cold; L. -a, -um, n. mare -is the sea; N.L. gen. n. *frigidimaris*, of a cold sea).

Cells were rod-shaped, 0.5 to 2.0 μm long and 0.5 to 0.8 μm wide, with cobweb like fibers on the cell surface. Non-motile. Cells occurred singly or as yellow-orange macroscopic aggregates. Growth occurred between 2 ℃ and 23 ℃, with an optimum at 16 ℃. Divided by binary fission. Did not grow on marine agar HaHa and marine agar 2216. With marine HaHa_min medium, growth occurred on casamino acids, peptone tryptone, yeast extract, gelatin, L-arabinose, D-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, N-acetyl-D-glucosamine, D-maltose, D-mannitol, D-mannose, N-acetylneuraminic acid, L-raffinose, L-rhamnose, D-sucrose, D-trehalose, DL-xylose, cellulose (structural affected filter paper), carboxymethyl cellulose, laminarin, agar, xanthan, κ-carrageenan and ι-carrageenan, but not on glycogen. Gelatin and xylan were not hydrolyzed. Vitamin requirement was not observed.

The strain, Hel1_33_78, was isolated from the seawater of Helgoland Roads, German Bight of the North Sea, Germany (54°11' N, 7°54' E).

Description of '*Polaribacter adhaesivus*'

'*Polaribacter adhaesivus*' (ad.hae'si.vum, N.L. masc. adj. *adhaesivus*, adhering, forming aggregates).

Cells were rod-shaped, 0.5 to 2.0 μm long and 0.5 to 0.8 μm wide, with fibrous mucus on the cell surface. Non-motile. Cells occurred singly or as yellow-orange macroscopic aggregates. Growth occurred between 0 ℃ to 26 ℃, with an optimum at 16 ℃. Divided by binary fission. Did not grow on marine agar HaHa and marine agar 2216. With marine HaHa_min medium, growth occurred on casamino acids, peptone tryptone, yeast extract, gelatin, L-arabinose, D-arabinose, D-cellobiose, D-fructose, D-glucose, D-galactose, N-acetyl-D-glucosamine, D-maltose, D-mannitol, D-mannose, L-raffinose, L-rhamnose, D-sucrose, D-trehalose, DL-xylose, glycogen, cellulose (structural affected filter paper), carboxymethyl cellulose, laminarin, agar, xanthan, κ-carrageenan and ι-carrageenan. Xylan was not hydrolyzed. Vitamins were not required.

The strain, Hel1_33_96, was isolated from the seawater of Helgoland Roads, German Bight of the North Sea, Germany (54°11' N, 7°54' E).

Description of '*Reinekea forsetii*'

'*Reinekea forsetii*' (for.set'ti.i. N.L. gen. fem. n. *forsetii*, of Forseti, a god in Scandinavian mythology that lived on Helgoland, the German island from where the bacterium was isolated).

Cells were non-pigmented, regularly coiled rods, 2 to 3 μm long and 0.4 to 0.5 μm wide. Motile by single polar flagella. In TEM pictures dark spots were observed, suggesting putative storage compounds. Growth occurred between 4 ℃ and 19 ℃, with an optimum at 12 ℃. Divided by binary fission. Generation time was 6 hours at 12 ℃. With marine HaHa_minV medium, growth occurred on casamino acids, peptone tryptone, yeast extract, gelatin, L-arabinose, D-arabinose, D-cellobiose, D-fructose, D-galactose, N-acetyl-D-glucosamine, D-glucose, D-maltose, D-mannitol, D-mannose, N-acetylneuraminic acid L-raffinose, L-rhamnose, D-sucrose, D-trehalose, DL-xylose and glycogen, but not on cellulose (structural affected filter paper), carboxymethyl cellulose, laminarin, agar, xanthan, κ-carrageenan and ι-carrageenan. Facultatively anaerobic; succinate, acetate, propionate, lactate and formate were produced under anaerobic conditions, from D-fructose and yeast extract, but not from D-galactose and N-acetyl-D-glucosamine. Vitamins were required.

The strain, Hel1_31_D35, was isolated from the seawater of Helgoland Roads, German Bight of the North Sea, Germany (54°11' N, 7°54' E).

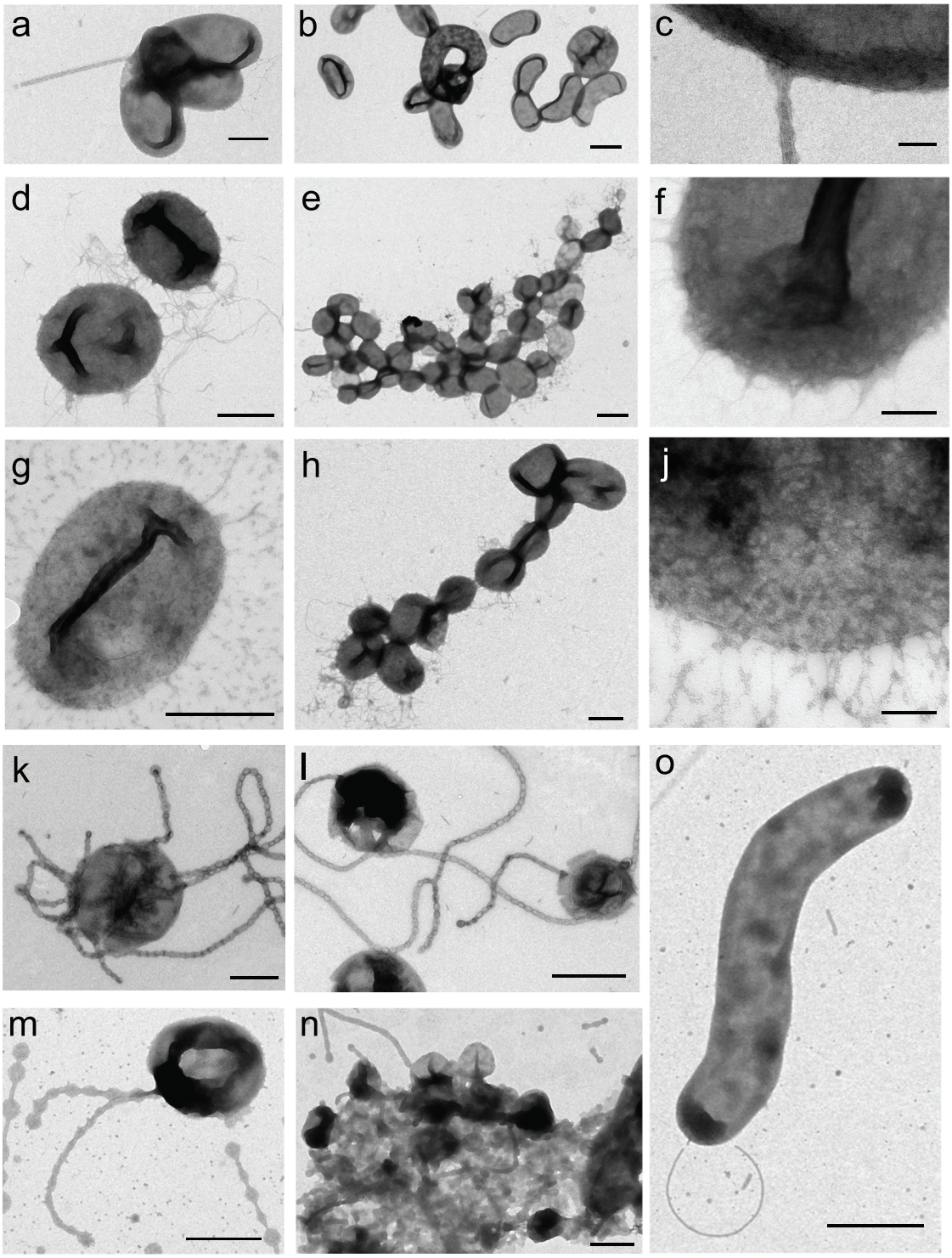

Figure 4.1 Cellular morphologies of marine strains documented by transmission electron microscopy images of **(a-c)** '***Polaribacter forsetii***', **(d-f)** '***Polaribacter frigidimaris***', **(g-j)** '***Polaribacter adhaesivus***', **(k, l)** '***Formosa flavarachnoidea***', **(m, n)** '***Formosa forsetii***', and **(o)** '***Reinekea forsetii***'. Depicted are single cells (a, d, g, k, m, o, bar 0.5 μm), aggregates (b, e, h, l, n, bar 1 μm), and cell surface structures (c, f, j, bar 100 nm) of strains.

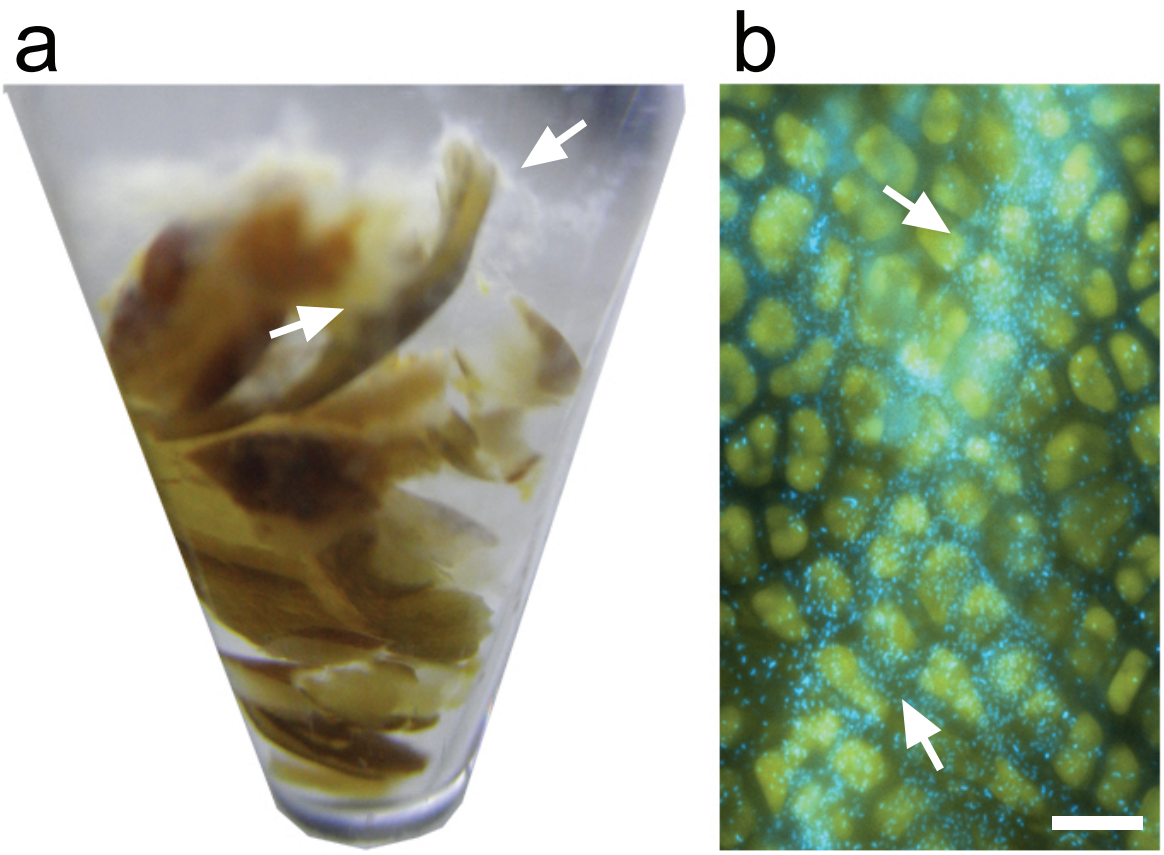

Figure 4.2 Documentation of growth on laminarin by *Polaribacter* and *Formosa* strains. (a) Macroscopic picture (Olympus, S7040) of a growing '*Polaribacter adhaesivus*' in culture tube visible by of formation of cloudy aggregates (arrow) on the surface of washed axenic *Laminaria saccharina* pieces. (b) Microphotograph (Axio Vision Camera, 400x magnification) of '*Formosa flavarachnoidea*' colonizing the surface of washed axenic *Laminaria saccharina* at affected sites (arrow). Green, auto-fluorescence; blue, DAPI stained bacterial cells; bar, 10 μm.

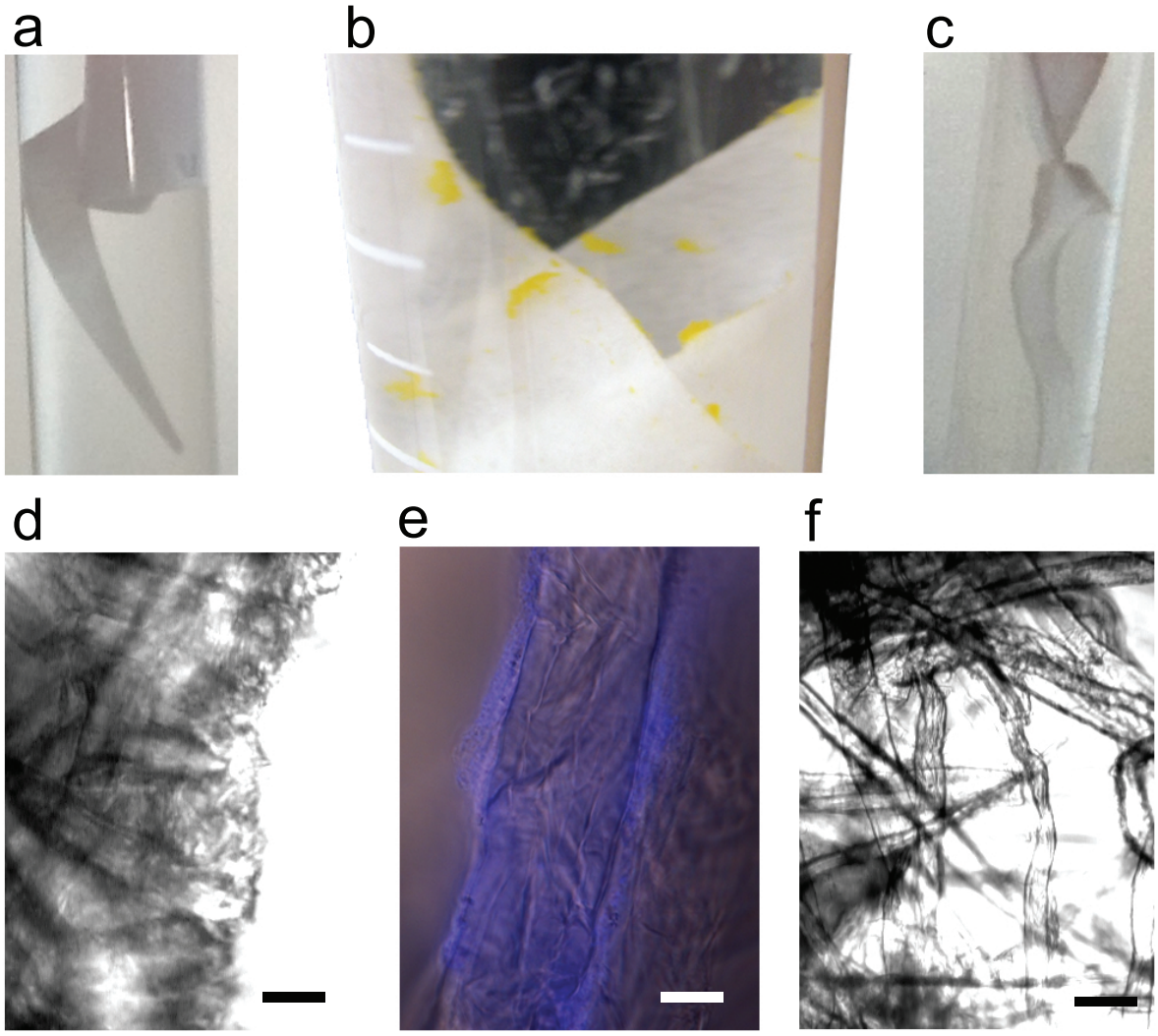

Figure 4.3 Documentation of growth on cellulose (structural affected filter paper) by *Polaribacter* (a, b, c) and *Formosa* (d, e, f) strains. Macroscopic pictures (a-c) were recorded with an Olympus S7040 camera and microphotographs were recorded with an Axio Vision Camera under the microscope at 400x magnification. Cellulose filter paper in medium without inoculum (a, d). Colonization and growth of '*Polaribacter adhaesivus*' on the cellulose filter paper (b; yellow spots, *Polaribacter* colonies) and '*Formosa flavarachnoidea*' on cellulose fibers (e; blue, DAPI stained cells). Affected filter paper by cellulose decomposing strains (c, f). All pictures were recorded after two weeks of incubation. White bar, 10 μm; black bar, 100 μm.

Table 4.1 Characteristics of *Formosa*, *Polaribacter*, and *Reinekea* strains aligned with type strains.

Strains: **1**, '*Formosa flavarachnoidea*'; **2**, '*Formosa forsetii*'; **3**, *Formosa agariphila* DSM 15362^T; **4**, *Formosa algae* KMM 3553^T; **5**, *Polaribacter* sp. Hel1_85; **6**, '*Polaribacter forsetii*'; **7**, '*Polaribacter frigidimaris*'; **8**, '*Polaribacter adhaesivus*'; **9**, *Polaribacter irgensii* 23-P^T; **10**, '*Reinekea forsetii*'; **11**, *Reinekea blandensis* MED297^T. For comparison, characteristics of *Formosa agariphila* DSM 15362^T, *Formosa algae* KMM 3553^T, *Polaribacter irgensii* 23-P^T and *Reinekea blandensis* MED297^T were shown as described in (Gosink et al., 1998; Ivanova et al., 2004; Nedashkovskaya et al., 2006; Pinhassi et al., 2007). Abbreviations: -, negative; w, slightly positive; +, positive; ++, strong positive; ND, not determined; *, grew on marine broth 2216; GlcNAc, N-acetyl-D-glucosamine; Neu5Ac, N-acetylneuraminic acid; CMC, Carboxymethyl cellulose.

Strains / Characteristics	*Formosa*				*Polaribacter*					*Reinekea*	
	1	2	3	4	5	6	7	8	9	10	11
Temperature for growth (°C):											
From	8	4	4	4	ND	2	2	0	-1.5	4	15
To	25	19	33	34	ND	22	23	26	17	19	42
Optimum	22	15	22	23	ND	14	16	16	12	12	ND
Vitamin requirement	+	−	−	−	−	−	−	−	−	+	−
Utilization of amino acids:											
Casamino acids	+	+	+	ND*	+	+	+	+	+	+	ND*
Peptone tryptone	+	+	+	ND*	+	+	+	+	ND*	+	ND*
Yeast extract	+	+	+	ND*	+	+	+	+	+	+	ND*
Gelatin	+	+	+	−	ND	+	+	+	−	+	−
Hydrolysis of gelatin:	+	+	+	w	ND	ND	−	+	−	+	−
Utilization of monosaccharides:											
D-galactose	+	−	+	−	+	−	+	+	+	+	w
D-mannose	−	−	+	−	+	+	+	++	+	+	+
L-rhamnose	−	−	+	−	−	−	+	+	−	+	−
D-fructose	−	−	+	−	+	−	+	+	−	+	+
D-glucose	−	+	+	+	−	+	++	++	+	+	+
D-mannitol	−	−	+	−	+	−	+	+	ND	+	+
DL-xylose	−	−	+	ND	+	−	+	+	−	+	−
L-arabinose	−	−	+	−	−	+	+	+	−	+	+
D-arabinose	−	−	+	−	−	+	+	+	−	+	+

Continued on next page

Table 4.1 continued

Characteristics \ Strains	*Formosa*				*Polaribacter*					*Reinekea*	
	1	2	3	4	5	6	7	8	9	10	11
Utilization of disaccharides:											
D-trehalose	−	−	+	−	−	−	+	+	−	+	−
D-sucrose	−	−	+	−	+	−	+	+	−	+	+
D-maltose	−	−	+	−	+	+	+	++	−	+	+
D-cellobiose	−	+	ND	−	−	+	+	++	−	+	+
Utilization of trisaccharides:											
L-raffinose	−	−	+	−	−	+	+	+	−	+	ND
Utilization of:											
GlcNAc	−	−	++	−	−	−	++	+	−	++	+
Neu5Ac	ND	ND	ND	ND	ND	ND	ND	ND	ND	++	ND
Utilization of polysaccharides:											
Cellulose (filter)	+	−	−	−	+	+	+	+	−	−	ND
Cellulose (CMC)	+	−	−	ND	+	+	+	+	ND	−	ND
Laminarin	+	+	+	ND	+	+	+	+	ND	−	ND
Agar	−	−	−	−	+	+	+	+	ND	−	−
Xanthan	w	−	w	ND	+	+	+	+	ND	w	ND
κ-carrageenan	w	w	w	ND	+	+	+	+	ND	−	ND
ι-carrageenan	−	w	−	ND	+	+	+	+	ND	−	ND
Glycogen	w	−	w	−	++	+	−	+	ND	+	ND
Hydrolysis of xylan	+	−	+	ND	−	−	−	−	ND	−	ND

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References

Arnosti, C. (2010). Microbial extracellular enzymes and the marine carbon cycle. *Annu Rev Mar Sci* **3**, 401–425.

Azam, F. (1998). Microbial control of oceanic carbon flux: The plot thickens. *Science* **280**, 694–696.

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H., Knaust, F. et al. (2006). Whole genome analysis of the marine bacteroidetes '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**, 2201–2213.

Cho, J. C., Vergin, K. L., Morris, R. M. and Giovannoni, S. J. (2004). *Lentisphaera araneosa* gen. nov., sp. nov., a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, *Lentisphaerae*. *Environ Microbiol* **6**, 611–621.

Choi, A. and Cho, J.-C. (2010). *Reinekea aestuarii* sp. nov., isolated from tidal flat sediment. *Int J Syst Evol Microbiol* **60**, 2813–2817.

Cottrell, M. T. and Kirchman, D. L. (2000). Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66**, 1692–1697.

Gerdts, G., Wichels, A., Döpke, H., Klings, K. W., Gunkel, W. and Schütt., C. (2004). 40-year long-term study of microbial parameters near Helgoland (German Bight, North Sea): historical view and future perspectives. *Helgol Mar Res* **58**, 230–242.

Gómez-Pereira, P. R., Schüler, M., Fuchs, B. M., Bennke, C., Teeling, H., Waldmann, J., Richter, M., Barbe, V., Bataille,

E., Glöckner, F. O. et al. (2012). Genomic content of uncultured *Bacteroidetes* from contrasting oceanic provinces in the North Atlantic Ocean. *Environ Microbiol* **14**, 52–66.

Gosink, J. J., Woese, C. R. and Staley, J. T. (1998). *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov., and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group and reclassification of '*Flectobacillus glomeratus*' as *Polaribacter glomeratus* comb. nov. *Int J Syst Bacteriol* **48**, 223–235.

Hahnke, R. L., Bennke, C. M., Fuchs, B. M., Mann, A. J., Teeling, H., Amann, R. and Harder, J. (2014). Dilution cultivation of marine heterotrophic bacteria benefiting from a coastal diatom bloom. *Environ Microbiol* **in prep.**

Ivanova, E. P., Alexeeva, Y. V., Flavier, S., Wright, J. P., Zhukova, N. V., Gorshkova, N. M., Mikhailov, V. V., Nicolau, D. V. and Christen, R. (2004). *Formosa algae* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *Int J Syst Evol Microbiol* **54**, 705–711.

Nedashkovskaya, O. I., Kim, S. B., Vancanneyt, M., Snauwaert, C., Lysenko, A. M., Rohde, M., Frolova, G. M., Zhukova, N. V., Mikhailov, V. V., Bae, K. S. et al. (2006). *Formosa agariphila* sp. nov., a budding bacterium of the family *Flavobacteriaceae* isolated from marine environments, and emended description of the genus *Formosa*. *Int J Syst Evol Microbiol* **56**, 161–167.

Pinhassi, J., Pujalte, M. J., Macian, M. C., Lekunberri, I., Gonzalez, J. M., Pedros-Alio, C. and Arahal, D. R. (2007). *Reinekea*

blandensis sp. nov., a marine, genome-sequenced gammaproteobacterium. *Int J Syst Evol Microbiol* **57**, 2370–2375.

Pinhassi, J., Sala, M. M., Havskum, H., Peters, F., Guadayol, O., Malits, A. and Marrase, C. (2004). Changes in bacterioplankton composition under different phytoplankton regimens. *Appl Environ Microbiol* **70**, 6753–6766.

Riemann, L., Steward, G. F. and Azam, F. (2000). Dynamics of bacterial community composition and activity during a mesocosm diatom bloom. *Appl Environ Microbiol* **66**, 578–587.

Schäfer, H., Bernard, L., Courties, C., Lebaron, P., Servais, P., Pukall, R., Stackebrandt, E., Troussellier, M., Guindulain, T., Vives-Rego, J. et al. (2001). Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in the genetic diversity of bacterial populations. *FEMS Microbiol Ecol* **34**, 243–253.

Smith, D. C., Steward, G. F., Long, R. A. and Azam, F. (1995). Bacterial mediation of carbon fluxes during a diatom bloom in a mesocosm. *Deep-Sea Res Pt II* **42**, 75–97.

Souza, C. P., Burbano-Rosero, E. M., Almeida, B. C., Martins, G. G., Albertini, L. S. and Rivera, I. N. G. (2009). Culture medium for isolating chitinolytic bacteria from seawater and plankton. *World J Microbiol Biotechnol* **25**, 2079–2082.

Tada, Y., Taniguchi, A., Nagao, I., Miki, T., Uematsu, M., Tsuda, A. and Hamasaki, K. (2011). Differing growth responses of major phylogenetic groups of marine bacteria to natural phytoplankton blooms in the western North Pacific Ocean. *Appl Environ Microbiol* **77**, 4055–4065.

Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J. et al. (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336**, 608–611.

Valentin, R. C., Shapiro, B. M. and Stadtman, E. R. (1968). Regulation of glutamine synthetase. XII. Electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry* **7**, 2143–2152.

Vimr, K. A., E. R.and Kalivoda, Deszo, E. L. and Steenbergen, S. M. (2004). Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev* **68**, 132–153.

Widdel, F. and Bak, F. (1992). *The Prokaryotes Vol 2*, chapter Gram-negative mesophilic sulfate-reducing bacteria. Balows A., Trüper H.G., Dworkin M. and Harder W. (eds), pp. 3352–3378. Springer, Berlin, Germany.

Winkelmann, N. and Harder, J. (2009). An improved isolation method for attached-living *Planctomycetes* of the genus *Rhodopirellula*. *J Microbiol Meth* **77**, 276–284.

Chapter 5

Conclusion and Discussion of the present work

The studies described in this thesis contribute to the cultivation of aerobic heterotrophic marine bacteria. The second chapter demonstrated the cultivation of marine *Flavobacteriaceae* on agar plates. The new HaHa medium and the new *Flavobacteria-Cytophagia* specific PCR assay enabled the targeted cultivation of marine *Flavobacteriaceae*. This collection of 375 *Flavobacteriaceae* strains from different habitats of the North Sea comprised the broad phylogenetic diversity of seven novel candidate genera, 42 novel species in 22 genera, and strains that were so far not distinguishable from 37 described species in 18 genera (*Chapter 2*).

Further investigations focused on the cultivation of pelagic bacteria that were of ecological relevance as representative key species during coastal diatom-dominated phytoplankton blooms in the North Sea (*Chapter 3*). Dilution cultivation of pelagic seawater and the new oligotrophic liquid HaHa medium led to a culturability of 35% of the bacteria counted in the same plankton sample by fluorescence *in situ* hybridization (FISH). Novel *Flavobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, and *Actinobacteria* isolates were obtained exhibiting of up to 99.8% sequence identities when compared to proteorhodopsin and full-lengths 16S rRNA gene

sequences of bacterioplankton of spring 2009. Using sequence-based comparisons of isolates draft genomes with metagenomes of bacterioplankton from spring 2009 we could show that reads of $\geqslant 95\%$ nucleotide identity covered the draft genomes of *Formosa* sp. by 94%, *Reinekea* sp. by 90% and *Polaribacter* sp. by 50%.

The fourth chapter investigated the physiological characteristics and carbohydrate substrate specificity of these representative key species. The results provided further insights into the capabilities of these novel species during the successive decomposition of algae derived polymers (*Chapter 4*).

Figure 5.1 ***(facing page)*** Phylogenetic relationship among isolates obtained in this thesis, type strains and lineages without cultured representatives of the family *Flavobacteriaceae*. The phylogenetic tree is based on comparisons of 16S rRNA gene sequences using the neighbour-joining method and a 0% and 40% base frequency filter of *Bacteroidetes*. Type strains of the classes *Bacteroidia*, *Cytophagia*, and *Sphingobacteria* were used as outgroups. The isolation source is indicated by: o, freshwater; ∼, marine environment; = terrestrial environment; +, clinical samples. Genera from which isolates were obtained from are color-coded: blue, from agar plates only; red, dilution cultures only; purple, both agar plates and dilution cultures; green, isolates that represent novel genera. *Flavobacteria* clades which had so far no representative culture are indicated by VIS (Gómez-Pereira et al., 2010), NS (Alonso et al., 2007) and DE (Kirchman et al., 2003). Scalebar represents 5 nucleotide substitutions per 100 nucleotides.


Bacteroidia/Cytphagia/Sphingobacteria
Maribacter
Pibocella
TBL_98_399
Muricauda
Flagellimonas
Pseudozobellia
Euzebyella
Croceitalea
Costertonia
Krilgaella
Zobellia
Arenibacter
Eudoraea
Zeaxanthinibacter
Muricola
Robiginitalea
Cellulophaga II
Cellulophaga I
Sediminicola
NS3a
Aequorivita
Vitellibacter
NS3b
Marixanthomonas
Hel1_48
Crocebacter
Leeuwenhoekiella
Gramella
Salegentibacter
Zunongwangia
Mesonia
Psychrophlexus
Salinimicrobium
Gillisia
Bizionia
MAR_2010_119
Gelidibacter
Subsaxibacter
Subaximicrobium
Olleya
Lacinutrix
Mesoflavibacter
Psychroserpens
Winogradskyella
Sediminibacter
MAR_2010_188
Hel1_10
Hyunsooneella
Tamlana
Jejuia
Algibacter
Flaviramulus
Mariniflexile
Snuella
Yeosuana
Gaetbulibacter
Gaetbulimamibacter
MAR_2010_10
MAR_2010_118
NS2b/VIS4
Ulvibacter
Gilvibacter
VIS5
Krokinobacter
Aquimarina
Dokdonia_Aquimarina
Capnocytophaga
Coenonia
Galbibacter
Zhouia
Joostella
Lutaonella
Leptobacterium
Gangjinia
DE2/NS5
VIS1
NS4/VIS2
Kordia
Fulvibacter
Tenacibaculum
Hel1_33_7
Polaribacter
Hel1_33_5
Lutibacter
Aestuariicola
Lutimonas
Actibacter
Flavobacterium
Myroides
Nonlabens
Empedobacter
Weeksella
Wautersiella
Ornithobacterium
Elizabethkingia
Soonwooa
Planobacterium
Riemerella
Cloacibacterium
NS1 marine group
Chryseobacterium
NS2a/VIS3
Formosa
freshwater
marine
terrestric
clinical
0.05

5.1 Improved cultivation of heterotrophic marine bacteria

The success of cultivation experiments can be affected by different factors, such as time point and location of sampling, sample preparation, incubation conditions, medium or the targeted bacteria. Different aspects that mainly contributed to the cultivation of novel species were discussed in *Chapter 2* and *Chapter 3* and will be combined and extended here.

Eilers (2000) and Eilers and colleagues (2001) combined the cultivation of marine pelagic bacteria on agar plates and the determination of their *in situ* abundance in the North Sea. They showed that members of *Cytophagia-Flavobacteria* had abundances of 18% of the bacterioplankton in winter and 30% in summer. The number of *Cytophagia-Flavobacteria* colonies was 12% of the 172 obtained colonies. Already after 15 days of incubation at 16 ℃ the final number of *Flavobacteria* colonies was reached and after 36 days new colonies were not observed (Eilers et al., 2001). Stevens and colleagues (2009) aimed at the cultivation of polymer degrading bacteria from one sampling station in the East Frisian Wadden Sea in fall, from different habitats such as seawater, aggregates and surface sediment. Most of the isolated bacteria were affiliated with *Actinobacteria* and *Gammaproteobacteria*. The low culturability of *Flavobacteria* in both studies is prompting the question whether marine members of *Bacteroidetes* can be cultivated on agar plates (Eilers et al., 2000b; Stevens et al., 2009). As demonstrated in *Chapter 2*, *Flavobacteria* can be cultivated from different pelagic and benthic samples of the North Sea, collected in spring, summer and fall. Moreover, these 375 *Flavobacteria* isolates represented 79 species in 33 marine genera (Fig. 5.1). It was also shown that incubation times of more than 15 to 39 days at 12 ℃ (*in situ* 6.4 ℃) were important for 80% of the *Flavobacteria* to form visible CFU on the agar plate (*Chapter 4*, Fig. 2.2 on

page 75) and that 20% of all colony forming units comprised *Flavobacteria*.

The short time of 30 min for transportation at *in situ* temperature, in contrast to the five hours transport on ice by Eilers and colleagues (2001) and Stevens and colleagues (2009), may have been advantageous to the cultivation of *Flavobacteriaceae*. In an enrichment experiment by Eilers and colleagues (2000a) it was shown that readily culturable *Vibrio* and *Alteromonas* rapidly increased in cell numbers, whereas the *Roseobacter* population remained constant. Further, they showed that members of *Vibrio* and *Alteromonas* maintain large amounts of cellular ribosomes during starvation (Eilers et al., 2000a) and thus they maintain a high potential for growth during starvation and can immediately respond to environmental changes (Flärdh et al., 1992).

The growth of bacteria of interest can be affected when exposed to stress factors during transport and cultivation (temperature shock, high concentration of a substance). A wide spread example is prophages induced cell lysis (Weinbauer et al., 2003). Although cells are protected during starvation, they are subjected to prophage induction as soon as they return to normal growth (Pearl et al., 2008). Prophages and phage infected cells were found in 10% to 90% of the bacterioplankton cells in the Gulf of Mexico and in the Mediterranean Sea and the Baltic Sea (Weinbauer and Suttle, 1999; Weinbauer et al., 2003). Alternatively, stressful cultivation conditions can trigger maintenance or dormancy of bacteria (Kaprelyants et al., 1993).

Flavobacteria are specialized for the decomposition of complex organic matter and it was reported several times that they preferentially utilize proteins and carbohydrates (Cottrell and Kirchman, 2000; Kirchman, 2002; Teeling et al., 2012; Fernández-Gómez et al., 2013). In previous studies, it was attempted to cultivate *Flavobacteria* on synthetic seawater medium supplemented with either a mixture of amino acids (Eilers et al., 2000b), monosaccharides (Eilers et al., 2000b) or polysaccharides (Stevens et al.,

2009). As mentioned above, on these media only a low number of *Flavobacteria* were cultivated. We suggested that for the cultivation of *Flavobacteria* carbohydrates and peptides should be available concurrently. Thus, we defined the new synthetic seawater HaHa medium which comprised carbohydrates (glucose, cellobiose) and peptides (yeast extract, peptone, casamino acids). The observed auxotrophy for amino acids of *Polaribacter* and *Formosa* strains corroborate our assumption (*Chapter 4*). This topic is discussed in more detail in section 5.2 *Streamlined genomes*.

The identification of interesting candidates among a complex community (enrichment culture) without clearly distinctive morphological features is challenging (Alain and Querellou, 2009). First, the diversity of morphological characteristics of the *Flavobacteriaceae* isolates ranges from bright yellow to dark brown colonies and from 0.6 μm long rods to 100 μm long filaments, as described for *Flavobacteriaceae* type strains (Bernardet, 2010). Second, strains of the genus *Polaribacter*, *Cellulophaga*, and *Tenacibaculum* were described to be polymorphic. Third, many strains of novel species and novel candidatus genera were not clearly distinguishable from other bacterial colonies by their morphological characteristics. This implies that on the one hand the culture collection of Stevens et al. (2009) which was based on different morphological features had a reduced phylogenetic diversity among the strains ($<$ 30 16S rRNA sequence types among 129 strains) and on the other hand, *Flavobacteria* might have been overlooked. As shown previously, a molecular screen by 16S rRNA PCR or FISH can support the cultivation of novel bacteria that were found in 16S rRNA clone libraries (Eilers et al., 2000b, 2001; Giebel et al., 2011) and can lead to a taxon specific cultivation (Winkelmann and Harder, 2009). Therefore, we used a specific PCR assay for the identification of *Flavobacteriaceae* among the colonies as straightforward method.

Species identification was even more important in our dilution cultivation

approach (*Chapter 3*). Button and colleagues (1993) developed the dilution cultivation and described (i) the preparation of pure cultures starting with a single cell as inoculum and (ii) the probability to obtain enrichment cultures from lower dilutions (randomly mixed bacterial populations). While the phylogenetic affiliation of pure cultures can be determined by 16S rRNA sequencing, the investigation of enrichment cultures depends on tools for bacterial community analysis such as clone libraries or FISH. In the targeted cultivation of *Reinekea* (*Chapter 3*) we combined the qualitative and quantitative detection of *Reinekea* in our enrichment cultures using a *Reinekea* specific PCR assay, followed by specific FISH on positive enrichment cultures. For future cultivation approaches I suggest to apply molecular screening methods for taxon identifications more frequently, as with the development of further taxon specific oligonucleotides –using the *rRNA approach* (Amann et al., 1995)– more representatives of hitherto uncultured taxa can be identified during cultivation experiments.

One of the major improvements for the cultivation of North Sea bacteria was the switch from cultivation on agar plates to dilution cultivation in oligotrophic liquid medium. This approach yielded a culturability of 35% of the bacterioplankton of Helgoland in spring 2010 (*Chapter 3*), in contrast to the culturability below 1% on agar plates (*Chapter 2*). The direct comparison was possible, because aliquots of the same seawater sample were either incubated on agar plates or in liquid medium. The dilution cultivation approach with the seawater of Helgoland in summer 2010 yielded a culturability of 30% of the bacterioplankton. Since the publications of Bere (1933) and of Jannasch and Jones (1959) we know that cultivation on agar plates leads to the 'great plate count anomaly' which points to the observation that only 1% of the natural bacterial community –determined by direct cell counting– can be cultivated on agar plates. For cultures on plates it was shown that (i) they might represent bacterial taxa of low abundance

(Eilers et al., 2000b) and (ii) they do not represent the same species as were found in clone libraries of the same habitat (Suzuki et al., 1997), based on the species boundary of 98.6% 16S rRNA sequence identity (Stackebrandt and Ebers, 2006). For example, Fig. 5.1 shows *Flavobacteriaceae* strains that were isolated on agar plates affiliated with diverse genera, but did not affiliate with *Flavobacteriaceae* clades that were found in clone libraries of the North Sea (Alonso et al., 2007) or the open ocean (Kirchman et al., 2003; Gómez-Pereira et al., 2010). On the contrary, isolates of dilution cultivation in oligotrophic seawater had 16S rRNA sequence identities of more than 99.8% with environmental clones of the same sampling location in the North Sea in spring 2009. Moreover, the 16S rRNA sequence of '*Formosa flavarachnoidea*' clustered within the NS2a and VIS3 clade of so far uncultured *Flavobacteria* which were found in the pelagic seawater of Helgoland in the North Sea (NS) (Alonso et al., 2007) and of the northern oceanic provinces in the North Atlatic Ocean (VIS) (Gómez-Pereira et al., 2010). A 16S rRNA sequence identity of more than 98.6% is an insufficient criterion to prove that these strains shared similar phenotypic and genomic characteristics with the corresponding *in situ* abundant species (Rosselló-Mora and Amann, 2001; Stackebrandt and Ebers, 2006). However, we applied the approaches of Konstantinidis and colleagues (Konstantinidis and Tiedje, 2005; Konstantinidis and DeLong, 2008) and thus could prove that '*Reinekea forsetii*' and '*Formosa forsetii*' formed populations of high genomic coherence with the environmental DNA of the spring 2009 bacterioplankton. These species were covered by 90% to 94% by metagenomic sequences of more than 94% nucleotide identity. Both criteria, an average nucleotide identity of more than 94% (Konstantinidis and Tiedje, 2005) and genomic convergence of 94% to 96% (Konstantinidis and DeLong, 2008) were suggested to circumscribe genetically coherent species.

Even though, some cultures from agar plates or dilution cultivation affili-

ate with the same genera *Polaribacter*, *Lutibacter*, and *Nonlabens* (Fig. 5.1), these strains do not belong to the same species (< 98% 16S rRNA sequence identity). This raises the general question whether agar plate isolates are genetically and metabolically different to those isolated by dilution cultivation. Three strains that originated from the same seawater sample may represent potential exemplary examples. Strains Hel1_29 and Hel1_41 grew on agar plates whereas strain Hel1_33_143 was isolated by dilution cultivation and did not grow on agar plates. These strains fall in the genus *Gillisia* and had a mutual 16S rRNA sequence identity of 100%. We could show that the inability to grow on agar plates was not caused by the presence of solid agar, the presence of EDTA-complexed trace elements or carbon concentrations as high as 1 g/L (*Chapter 3*). However, one phenotypic trait differed among these strains. The dilution culture Hel1_33_143 and all other dilution cultures could not cope with 2 mM or 50 mM of HEPES buffer in the medium. It is not clear whether potential toxic effects originated from phototoxicity (Zigler et al., 1985), the formation of radical species (Grady et al., 1988), or an increased bioavailability of copper (Lage et al., 1996). Jannasch and Mateles (1974) could show that bacterial populations of low cell density were unable to cope with well oxygenated medium in seawater and freshwater chemostats, but bacterial populations of high cell density could overcome the high oxygen concentration. With increasing cell densities the total respiratory capacity of the community increases and possibly prevents cellular damage caused by oxidative stress (Krieg and Hoffman, 1986). However, Bruns and colleagues (2003) could not increase the bacterioplankton culturability by reducing the oxygen partial pressure to 3%, possibly because microaerophilic or anaerobic bacteria did not constituted a major fraction of the bacterioplankton.

Previous high-throughput cultivation studies aimed at the isolation of individual bacterioplankton cells by diluting the seawater sample to near extinction (Zengler et al., 2002). In contrast to these studies, our dilution cultivation approach included the cultivation of mixed bacterioplankton populations by diluting the seawater sample to approximately 10 or 100 cells per inoculum. Three results led to the assumption that this approach enabled us to prepare by chance a favorable bacterial community in the dilution culture Hel1_31_5 which potentially promoted the growth of '*Reinekea forsetii*'. First, '*Reinekea forsetii*' grew in the vitamin free HaHa medium although this species was auxotroph for vitamins. Second, once we supplemented the HaHa medium with vitamins a separation of '*Reinekea forsetii*' was possible using consecutive dilution cultivation. Third, the bacterial community of the dilution culture Hel1_31_5 was dominated by *Polaribacter* which were previously shown to succeed simultaneously with *Reinekea* during the spring phytoplankton bloom in the North Sea (Teeling et al., 2012). This hypothetical interaction of *Reinekea* and *Polaribacter* needs further investigation to clarify whether (i) cross-feeding between both species is possible and (ii) the co-occurrence in culture and *in situ* is a general characteristic.

The possibility to generate large numbers of co-cultures of random mixed bacterial communities was described by Button and colleagues (1993), but so far have not been widely implemented in the cultivation of marine bacteria. Co-cultivation of bacterial populations might enable 'helper' organisms to promote the growth of as yet uncultured bacteria (D'Onofrio et al., 2010). For example, *Alteromonas* depleted hydrogen peroxide in the medium and thereby reduced the oxidative damage of hydrogen peroxide in *Prochlorococcus* (Morris et al., 2011). D'Onofrio et al. (2010) showed the positive effect of siderophores secreted by *Micrococcus* (*Actinobacteria*) and *Vibrio* (*Gammaproteobacteria*) colonies promoting the growth of neighboring *Flavobacteriaceae* and *Gammaproteobacteria*. For future cultivation

both perspectives of dilution cultivation can help to cultivate as yet uncultured marine bacteria and may offer interesting insights into the interaction of bacterial communities. Further aspects of metabolic dependency are discussed in the next section 5.2 *Streamlined genomes*.

5.2 Streamlined genomes

Morris and colleagues (2012) hypothesized an evolutionary adaptation towards a metabolic dependency between bacterioplankton populations yielding a higher fitness of the overall bacterioplankton community. They proposed that a 'helper' population has a broad spectrum of metabolic functions and is leaky for public goods (e.g. vitamins, siderophores), whereas a 'beneficiary' population lost metabolic functions in consequence of selective genome reduction. As a consequence, the population size of the 'beneficiary' population would be regulated by the 'helper' populations that provide the desired compounds.

So far, the 2.75 Mbp genome of *Polaribacter irgensii* 23-P^{T} and the 2.97 Mbp genome of *Polaribacter doktonensis* MED152 were reported as the smallest genomes among the *Bacteroidetes* (González et al., 2008), the 1.75 Mbp genome of *Prochlorococcus marinus* SS120^{T} as the smallest genome among cyanobacteria (Dufresne et al., 2003), the 1.27–1.7 Mbp draft genomes of members of the *Gammaproteobacteria* lineage SAR86 (Dupont et al., 2012) and the 1.3 Mbp genome of '*Candidatus* Pelagibacter ubique' (Giovannoni et al., 2005) as the smallest genomes among free-living bacteria. The draft genomes of the obtained isolates '*Polaribacter forsetii*' and '*Formosa forsetii*' (*Formosa* clade B) had a size of 2.99 Mbp and 2.75 Mbp, comparable to the genome size of *Polaribacter irgensii* 23-P^{T} and *Polaribacter doktonensis* MED152. Moreover, the draft genome of the isolate '*Formosa flavarachnoidea*' (*Formosa*clade A) had a size of 2.01 Mbp.

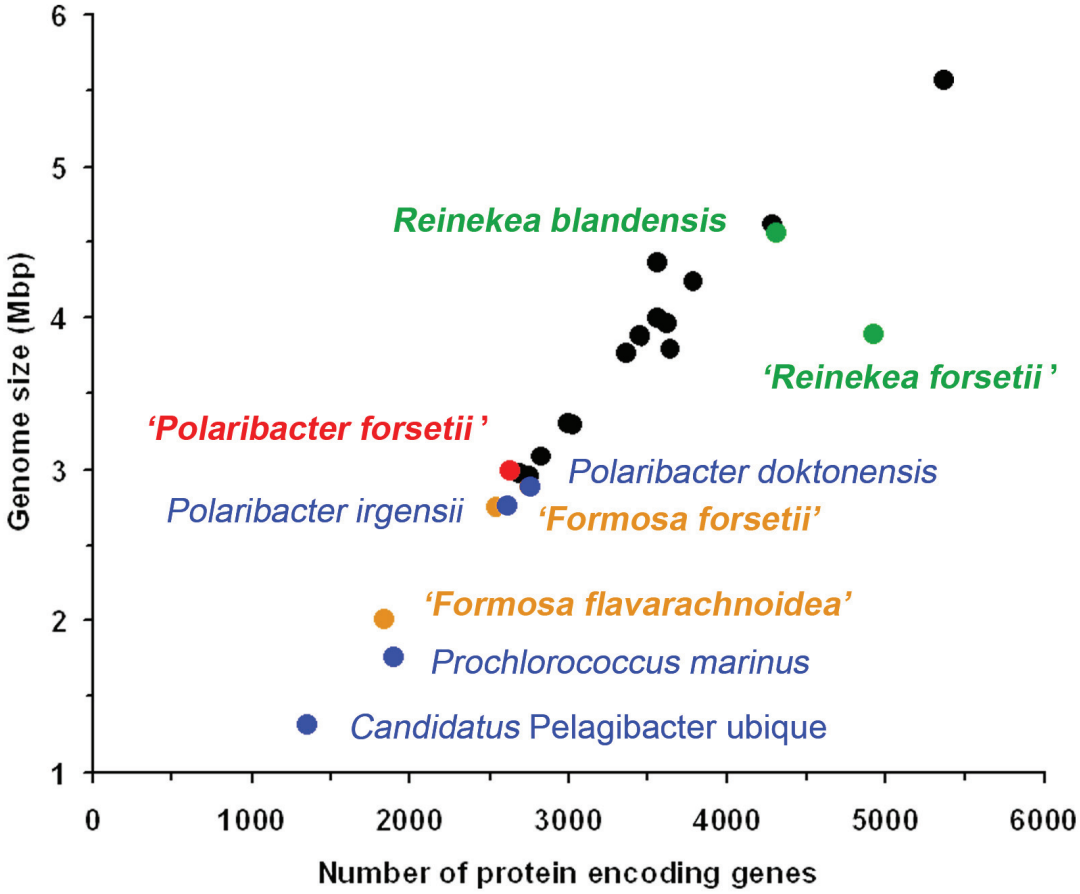

Figure 5.2 Number of predicted protein-encoding genes versus genome size for genomes of isolates, genome sequenced *Flavobacteriaceae* type strains (gray dots), and genome streamlined bacteria '*Candidatus* Pelagibacter ubique' HTCC1062 (Giovannoni et al., 2005), *Prochlorococcus marinus* SS120^{T} (Dufresne et al., 2003), *Polaribacter doktonensis* MED152 (González et al., 2008) and *Polaribacter irgensii* 23-P^{T} (acc. AAOG00000000).

Thus, this isolate has the smallest genome of all so far sequenced *Bacteroidetes* genomes. The reduced genome sizes of the *Polaribacter* and *Formosa* isolates were the result of a reduction of protein-coding genes compared to other genome-sequenced *Flavobacteria* (Fig. 5.2).

Such a small genome might indicate a genomic and metabolic streamlining as shown for '*Candidatus* Pelagibacter ubique' HTCC1062 (Giovannoni et al., 2005) and *Prochlorococcus marinus* SS120^{T} (Dufresne et al., 2003, 2005), *Polaribacter doktonensis* MED152 (González et al., 2008) and mem-

bers of the *Gammaproteobacteria* lineage SAR86 (Dupont et al., 2012). The genome streamlining of '*Candidatus* Pelagibacter ubique' HTCC1062 caused a metabolic dependency on pyruvate as carbon source, methionine as sulfur source, and glycine or serine as essential amino acids (Carini et al., 2012). This was the reason that for a long time '*Candidatus* Pelagibacter ubique' HTCC1062 could only be cultivated in natural seawater based medium, and that it grew to varying cell densities and with varying growth rates depending on the source of the seawater (Rappé et al., 2002). In *Prochlorococcus marinus* SS120^{T} the small genome resulted from the deletion or underrepresentation of genes involved in DNA repair, transporters and metabolism (Dufresne et al., 2003, 2005). As a major consequence, *Prochlorococcus marinus* SS120^{T} is missing urea, nitrate and nitrite transporters and relies on the import of reduced nitrogen compounds such as ammonia and amino acids (Dufresne et al., 2003). *Polaribacter doktonensis* MED152 was unable to use fermentation or anaerobic respiration for energy conservation and thus can only perform aerobic respiration. Furthermore, as nitrogen and sulfur sources *Polaribacter doktonensis* MED152 was able to use only ammonia and sulfate (González et al., 2008). Members of SAR86 were predicted to be auxotroph for vitamins such as B_6, biotin and thiamine, and for amino acids such as methionine, histidine, and arginine (Dupont et al., 2012). Additionally, SAR86 genomes lacked the enzymes required for assimilatory sulfate reduction and uptake. Instead, SAR86 members likely require as sulfur source glutathione or dimethyl-sulfoniopropionate, compounds than can both be detected in surface seawater (Dupont et al., 2006; Reisch et al., 2011). We observed that the growth of the *Formosa* and *Polaribacter* cultures were dependent on the presents of amino acids (*Chapter 3*). It still needs to be determined which amino acids were essential for growth. Since these cultures grew on casamino acids (Bacto™) and peptone tryptone (Bacto™) and the applied casamino acids lack asparagine and glu-

tamine (see Tab. 3.S2 in Chapter 3 on page 154), it can be assumed that asparagine and glutamine were not essential for growth. It is known that *Flavobacteria* degrade dissolved organic matter (DOM) of high-molecular-weight (Kirchman, 2002) and it was shown that they utilize besides carbohydrates a significant amount of the co-occurring proteins (Cottrell and Kirchman, 2000; Teeling et al., 2012; Fernández-Gómez et al., 2013). Hence, *Flavobacteria* may have evolved a metabolic dependency on proteins, because both carbohydrates and proteins are available in environments were *Flavobacteria* prevail (Wakeham et al., 1997; Kirchman, 2002). We also observed that the *Formosa* sp. Hel3_A1_48 and *Reinekea* sp. Hel1_31_D35 strains required vitamins for growth. This was observed after some transfers in vitamin free medium or in cultures of higher cell density. Hence, traces of vitamins could have originated from the diluted surface seawater or the yeast extract. Auxotrophy for at least one B vitamin was shown to be common for bacterioplankton since they lack the biosynthetic pathways for the production of the vitamin (Sañudo-Wilhelmy et al., 2012). However, microorganisms presumably undergo such selective genomic and metabolic rearrangements to minimize the production of cellular components such as cell structures, proteins and DNA and thereby reduce the demand of carbon, nitrogen, phosphorus, and other nutrients which are particularly limited in marine environments (Dufresne et al., 2005; Giovannoni et al., 2005). The repeatedly observed small cell size leads to an improved nutrient uptake with an increasing cell surface-to-volume ratio (Button, 1991). As presented in *Chapter 3*, the *Formosa* and *Polaribacter* strains had a cell size of less that one and two micrometer, respectively. Teeling and colleagues (2012) could show that these bacterial populations rapidly increased in cell numbers after the phytoplankton bloom. Thus, a reduced genome, cell size and a limited metabolic repertoire might be an advantage during the bacterial succession in spring.

5.3 Proteorhodopsin

Proteorhodopsin is an integral membrane protein of bacteria which functions with retinal as a light-driven proton pump in the marine environment (Béjà et al., 2000, 2001). Proteorhodopsins were found in marine bacterioplankton of the Sargasso Sea (Venter et al., 2004), the Pacific Ocean (de la Torre et al., 2003; Yoshizawa et al., 2012), the Mediterranean Sea and the Red Sea (Man et al., 2003; Sabehi et al., 2005), the Antarctic (Béjà et al., 2002), the North Atlantic Ocean (Sabehi et al., 2005; Campbell et al., 2008), and the North Sea (Riedel et al., 2010; Teeling et al., 2012). Riedel and colleagues (2010) estimated that 50% of the bacterioplankton in the North Sea harbour proteorhodopsin of which the majority potentially absorbed green light.

We could show that the proteorhodopsin sequences of our *Flavobacteriaceae* isolates (Chapter 3) were $>$ 99% identical with (i) metagenome sequences of the bacterioplankton community in the North Sea in spring 2009 (Teeling et al., 2012), and likewise with (ii) proteorhodopsin clones of the North Sea in summer 2006 (Riedel et al., 2010), (iii) proteorhodopsin clones of the North Atlantic Ocean (Sabehi et al., 2005), or (iv) *Polaribacter* isolates from the sea ice of the Sea of Ochotsk, Hokkaido, Japan (Yoshizawa et al., 2012). Based on the single amino acid substitution which differentiates between the light absorption maximum at 490 nm (blue) and 540 nm (red) (Man et al., 2003), the corresponding proteorhodopsins were characterized as green light absorbing. This suggests that the *Polaribacter*, *Formosa*, and the candidate *Flavobacteriaceae* genus isolates might prevail in the surface seawater of the North Sea and other oceans, and that these strains were potentially adapted towards the light spectra of the surface seawater, where they were isolated from. Moreover, the close affiliation of the strain Hel1_33_7 with the phylogenetically uncharacterized proteorhodopsin clones NA13_R15_12 and NA11_R15_8 (Sabehi et al., 2005) reveals that these

clones can be affiliated with the novel candidate genus (represented by strain Hel1_33_7) of the family *Flavobacteriaceae*.

The function of proteorhodopsin is so far speculative, because the influence of light has not been investigated. Yoshizawa et al. (2012) showed for the culture *Polaribacter* sp. SA4-10 the activity of the light-driven proton pump translocating protons from the cytoplasm into the periplasm and thereby decreasing the pH of the surrounding medium by ΔpH 0.05. This suggested that based on the results of other investigators (Oesterhelt and Stoeckenius, 1973; Béjà et al., 2000) the resulting membrane potential might be sufficient for ATP synthesis. The proteorhodopsin of *Dokdonia donhaenensis* PRO95^{T} was constitutively expressed independent from the incubation in light or dark and the organic matter concentration, but growth stimulation by light could not be confirmed (Riedel et al., 2010). During starvation, the growth of *Dokdonia donhaenensis* PRO95^{T} and the survival of *Vibrio* sp. AND4 was enhanced by light (Gómez-Consarnau et al., 2007, 2010). González et al. (2008) showed that the *Flavobacteria* strain *Polaribacter doktonensis* MED152 did not live autotrophically with light, but needed organic carbon sources. Furthermore, the authors drew the conclusion that anaplerotic CO_2 fixation was stimulated by light, based on a higher CO_2 uptake in *Polaribacter doktonensis* MED152 under light than in the dark. They additionally proposed a biphasic life-style of *Polaribacter doktonensis* MED152. First, when complex organic matter is readily available these substrates serve as energy and carbon source and proteorhodopsin phototrophy might provide energy for the TonB-dependent transport of oligosaccharides. Second, under oligotrophic conditions light energy increases the anaplerotic CO_2 fixation to replenish the TCA cycle that intermediates can be used effectively for biosynthesis. For our *Polaribacter*, *Formosa*, and the candidate *Flavobacteriaceae* genus strains which were so far generally incubated in the dark, the proposed life-style might also apply.

5.4 Targeted isolation of polysaccharide binding bacteria

Carbohydrate binding modules (CBMs) specifically recognize oligosaccharide moieties and promote a prolonged interaction with the substrate (Boraston et al., 2004). CBMs are non-catalytic modules found in carbohydrate-active enzymes (Boraston et al., 2004; Cantarel et al., 2009). *Bacteroidetes* are specialized for the initial attack of complex organic material (Kirchman, 2002) and their genomes have been shown to encode for numerous carbohydrate-active enzymes (González et al., 2008; Martens et al., 2011; Gómez-Pereira et al., 2012; Teeling et al., 2012). Their strategy is rather the direct binding to the substrate than the secretion of extracellular carbohydrate active enzymes (Kirchman, 2002; Bauer et al., 2006).

Carbohydrates can either be directly coupled (Seljelid et al., 1985) or directly bound via antibodies and lectins (Sternemarr et al., 1992) on the surface of beads. However, to preserve the presentation of the defined carbohydrate moieties for the interaction with CBMs, carbohydrates are coupled to bovine serum albumin (BSA) yielding a neoglycoprotein (Roy et al., 1984). These neoglycoconjugates are spotted on nitrocellulose membranes of 0.45 μm pore size or nitrocellulose coated glass slides, as applied for carbohydrate microarrays (Pedersen et al., 2012). In contrast to BSA, streptavidin binds readily to polystyrene surfaces and when coupled to biotin it insignificantly interacts with proteins. Hence, an enrichment of carbohydrate binding microorganisms can be achieved with the help of biotin-streptavidin coated magnetic beads using either (i) lipids as spacer yielding neoglycolipids, or (ii) using polyacrylamide as spacer yielding pseudo-polysaccharides (Rye, 1996). For example, L-selectin coated beads were used to separate acute lymphoblastic leukemia cell lines (Rye and Bovin, 1997). As a consequence of the wide variety of different glycosidic bonds and nat-

urally occurring monosaccharides, oligosaccharides are chemically complex (Cantarel et al., 2009; Warren, 1996). The number of possible combinations in a linear and branched hexasaccharide composed of D-hexoses of the same molecular mass is larger than 10^{12} (Laine, 1994). This *Isomer Barrier*, precludes the determination of the oligosaccharide structure by sequencing methods like the Edman technique for peptides or the Sanger technique for DNA (Laine, 1994). Furthermore, the macromolecular structure of particulate and dissolved organic matter in the ocean is mostly unknown. It is also largely unknown which specific structures of the organic matter are remineralized in the seawater and sediments and which part remains as recalcitrant material (Lee et al., 2004). The initial approach would be the application of an already existing oligosaccharide library which was developed to analyze plant or algae cell walls (Pedersen et al., 2012). In a second approach, the beads could be coated with naturally available oligosaccharides, generated by the extraction and specific enzymatic or incomplete chemical hydrolysis of phytoplankton carbohydrates (Rye, 1996; Pedersen et al., 2012). Since the association constants for protein-carbohydrate interactions (K_a 10^3–10^4) are low in comparison to antigen-antibody reactions (K_a 10^3–10^9), the carbohydrate binding of the cell is reversible by the addition of the recognized oligosaccharides moiety (competitive reaction) (Rye and Bovin, 1997). Therefore, cells can be released after the first binding to the carbohydrate coated beads. A consecutive enrichment with a set of different oligosaccharides is possible, based on the high specificity of the CBMs towards the oligosaccharides (Rye and Bovin, 1997). This approach would allow the investigation of distinct bacterioplankton populations during the phytoplankton decomposition. The cultivation of single cells can be achieved by diluting the enriched and released bacterial population to near extinction. Alternatively, beads of nanometer scale (Wang et al., 2004) which bind only one cell, can be distributed into separated enrichments.

5.5 Sialic acid metabolism

Although the following is speculative, it allows considerations about possible interactions between bacterioplankton populations during the bacterial succession after the coastal phytoplankton bloom in spring. The genomes of '*Formosa flavarachnoidea*', '*Formosa forsetii*', '*Reinekea forsetii*', and '*Polaribacter forsetii*' enabled the screen for potential metabolic pathways that could explain niche differentiation. It should be mentioned here that the order of the strains named above represents the order of their successive blooming in spring 2009.

The *de novo* biosynthesis of neuraminic acid (Neu5Ac) is catalyzed by the enzymes UDP-GlcNAc epimerase (NeuC) and Neu5Ac synthase (NeuB) from UDP-GlcNAc, a common precursor of the cell wall (Fig. 5.3) (Vimr et al., 2004). UDP-GlcNAc is produced from fructose-6-P or glucosamine-6-P via glucosamine-6-P synthase (GlmS), phosphoglucosamine mutase (GlmM) and GlcNAc-1-P uridyltransferase (GlmU). Neu5Ac enters the common polysialic acid (PSA) biosynthetic pathway via CMP-Neu5Ac ligase (NeuA) and Neu5Ac O-acetyltransferase (NeuD). The polysialyltransferase (NeuS) adds Neu5Ac to oligosialic acid receptors to form the PSA capsule, which is then exported through the PSA capsule export system (Kps). The genes *NeuA*, *NeuB*, *NeuC*, *NeuD*, the regulator GntR and the Kps module were found to be co-localized in the genomes of *Formosa* and *Reinekea*. Thus, *Formosa* and *Reinekea* potentially build up their glycocalix with sialic acids. These genes were not found in the genome of *Polaribacter*, suggesting a different glycocalix composition.

Extracellular neuramidases (e.g. endo-α-(2,8)-sialidase) cleave glycosidic linkages of terminal sialic acid residues in oligosaccharides, glycoproteins or glycolipids (Fig. 5.3). The product Neu5Ac enters the cell via the specific ABC (ATP-binding cassette) transporter SatABCD, the

TRAP (tripartite ATP-independent periplasmic) transporter SiaPQM, or the MFS (major facilitator superfamily) transporter NanT (Almagro-Moreno and Boyd, 2009). Within the cell, neuraminic acid (Neu5Ac) is catabolized by enzymes of the NanAKEX and NagAB-GntR clusters. The catabolic pathway involves five steps (Fig. 5.3) (Almagro-Moreno and Boyd, 2009): N-acetylneuraminic acid lyase (NanA) removes the pyruvate group from Neu5Ac yielding N-acetylmannosamine (ManNAc). N-acetylmannosamine kinase (NanK) catalyzes the addition of a phosphate group to ManNAc yielding N-acetylmannosamin-6-P (ManNAc-6P). N-acetylmannosamin-6-P epimerase (NanE) epimerizes ManNAc-6P to N-acetylglucosamine-6-P (GlcNAc-6P). Finally, N-acetylglucosamine-6-P deacetylase (NagA) removes the acetyl group yielding glucosamine-6-P (GlcN-6-P). The amino group is removed by the glucosamine-6-P deaminase (NagB) yielding fructose-6-phosphate (Fru-6P) that enters the glycolysis.

Exclusively the genome of '*Reinekea forsetii*' contained the complete NanAKE and NagAB gene cluster, the corresponding regulators NanX and GntR, the extracellular endo-α-(2,8)-sialidase, and the Neu5Ac TRAP transporter SiaPQM. Interestingly, a significant number of glycoside hydrolases of the family 2 (GH2) were assigned to *Reinekea* in the bacterioplankton metagenome in spring 2009. The GH2 comprises β-glycosidases that catalyze the hydrolysis of glycosaminoglycans (Cantarel et al., 2009; Withers, accessed Dez. 2012). Glycosaminoglycans consist of an amino sugar (N-acetylglucoseamine or N-acetylgalactosamine) along with a uronic sugar (glucuronic acid or iduronic acid) or galactose (Esko et al., 2009). Interestingly, Teeling at al. (2012) showed that during the decomposition of the spring phytoplankton in 2009 the *Formosa* population diminished at the same time as the *Reinekea* and *Polaribacter* population started to bloom. The *Formosa* population spiked a second time once the *Reinekea* population decreased to less than 5%. These authors hypothesized that the

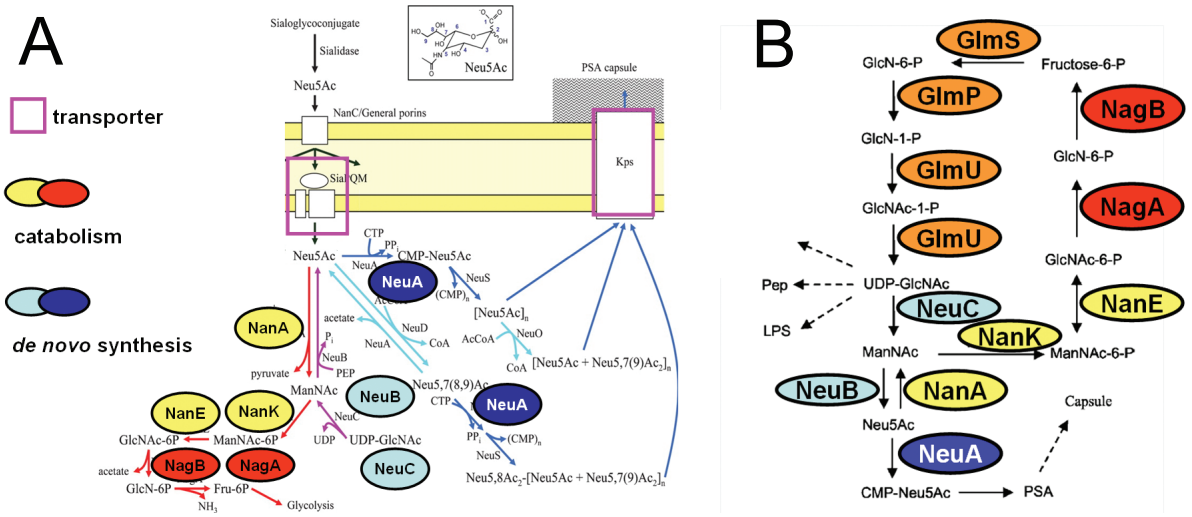

Figure 5.3 Proposed pathways of *de novo* sialic acid synthesis and catabolism. (A) Genes that are involved in the decomposition of sialic acid containing lipopolysaccharides (sialioglyco-conjugates) via the sialidase, import of acetyl-neuramic acid (Neu5Ac) via porins and the SiaPQM transporter, activation and isomerization to fructose-6-phosphate (fructose-6-P) via the NanAKE and NagAB cluster. Furthermore, genes for the *de novo* synthesis of Neu5Ac from the activated UDP-N-acetylglucosamine (UDP-GlcNAc) via the NeuBC cluster and glycopolysaccharides with Neu5Ac on the cell surface via NeuA and Kps. (B) Additionally to the genes for sialic acid utilization, genes are depicted that encode for the *de novo* synthesis of UDP-N-acetyl-glucosamine (UDP-Glc-NAc) from fructose-6-phosphate via the GlmSPU cluster. Modified after (Severi et al., 2007) and (Vimr et al., 2004).

swift succession of the bacterioplankton populations in spring 2009 was triggered by the availability of algae derived carbohydrates. Indeed, the phytoplankton composition shifted mainly from *Thalassiosira* to *Chattonella* and *Phaeocystis* (Teeling et al., 2012). The major carbohydrates produced by *Phaeocystis* species comprise polysaccharides of glucose, mannose, rhamnose, and sialic acid (Thingstad and Billen, 1994), whereas *Thalassiosira* species consist mainly of galactose, glucose, mannose, rhamnose and fucose (Urbani et al., 2005). This suggests that '*Reinekea forsetii*' can make use of the sialic acids containing glycocalix of *Formosa* and *Phaeocystis*, either for biosynthesis of its own polysialic acids or for utilizing acetyl-neuramic acid as potential carbon and nitrogen source. Indeed, I could show that

'*Reinekea forsetii*' grew on N-acetylneuraminic acid and the *Formosa* and *Polaribacter* strains did not (see *Chapter 4*).

However, Sañudo-Wilhelmy et al. (2012) suggested that besides the eukaryotic phytoplankton, bacterioplankton populations are effected by the availability of vitamins, because marine bacteria are quantitatively the most important consumers of vitamins as growth factors. Indeed, the *Reinekea* culture required vitamins for growth (see *Chapter 3*). Since algae and bacteria are sources of vitamins (Gobler et al., 2007) and *Polaribacter* together with *Reinekea* have been shown to coexist in the same habitat (Teeling et al., 2012), we proposed *Polaribacter* populations as a vitamin source during the co-cultivation with '*Reinekea forsetii*' (*Chapter 3*). Hence, algae or bacteria derived vitamins cannot be excluded as a trigger of the '*Reinekea forsetii*' bloom in spring 2009.

Conclusively, '*Reinekea forsetii*' utilized sialic acids and might have caused the decline of the *Formosa* population. *Polaribacter* was probably not effected by the sialic acid utilization of *Reinekea*. Instead, *Polaribacter* might have been a vitamin source for '*Reinekea forsetii*'. Certainly, co-cultivation studies of *Formosa*, *Reinekea*, and *Polaribacter* cultures and axenic cultures of *Thalassiosira* and *Phaeocystis* are important to elucidate specific interactions among the bacterioplankton populations and between bacterioplankton and phytoplankton populations.

5.6 Outlook

Since the strain collection obtained in this study contributes substantially to the diversity of discribed North Sea *Flavobacteriaceae*, the isolates should be taxonomically classified. The requirements must follow the minimal standards for describing taxa of the family *Flavobacteriaceae* (Bernardet et al., 2002). We already started to group strains based on whole-cell protein profiling by MALDI-TOF MS to reduce the number of strains in this collection beforehand. This approach has been recently reviewed in its potential as rapid tool for microbial identification and phenotyping (Moore and Rosselló-Móra, 2011).

The genomes of these *Flavobacteriaceae* isolates might encode for numerous polysaccharide utilization loci (PUL) and thus, genome sequencing of dedicated strains will give insights into arrangements, similarities and co-localization of carbohydrate active enzymes (CAZyme) and associated TonB-dependent transporters (for example see Tab. 5.1). However, the classification of CAZymes based on sequence similarity (Cantarel et al., 2009) has the consequence that enzymes are grouped together in gene families which may have quite different substrate specificies (Henrissat, 1991). Thus, a biochemical characterization of enzymes is needed for an unambiguous functional assignment (Henrissat, 1991). For example, the number of protein sequences in the database of CAZymes increased exponentially between 1999 and 2007, while the number of characterized enzymes increased only linearly (Cantarel et al., 2009). Moreover, it is largely unknown whether certain bacterioplankton clades that encode for PULs in their genome concertedly decompose complex polymeric carbohydrates (synergistic, functionally complementary) or utilize the same complex carbohydrate, but with different sets of enzymes (independent, functionally redundant). For example, the genomes of *Bacteroides thetaiotaomicron* and *B. ovatus* of the gut mi-

Table 5.1 Genome size, predicted ORF, carbohydrate active enzymes (CAZymes), TonB-dependend and ABC type transporter encoded in the genomes of the novel *Formosa*, *Polaribacter*, und *Reinekea* species.

	Formosa		*Reinekea*	*Polaribacter*	
	'*flavarachnoidea*'	'*forsetii*'	'*forsetii*'	'*forsetii*'	sp. Hel1_85
Contigs	17	1	79	31	63
total bases (Mb)	2.01	2.73	3.71	2.99	3.86
total ORFs	1,848	2,546	4,879	2,634	3,403
CAZymes	121	125	235	292	404
Glycosid hydrolases	38	36	66	90	110
Sulfatases	17	9	1	13	35
TonB dep. transporter	13	19	1	6	8
ABC transporter	16	16	65	11	12

crobiome encode for 28 homologous PULs for the utilization of glycans of the gut mucosa and plant cells (Martens et al., 2011). The niche speciation of both species was attributed to eight unique PULs of *B. thetaiotaomicron* to degrade O-glycans, and five unique PULs of *B. ovatus* that targeted hemicelluloses of the plant cell wall (Martens et al., 2011). It is also possible that different sets of glycoside hydrolases at the outer membrane decompose complex carbohydrates to common oligosaccharides which are then transported into the periplasm and hydrolyzed to monosaccharides by the same set of CAZymes. The expression of PUL-genes is regulated by the TonB-dependent sensor that directly interacts with linear oligosaccharides. Hence, related glycans composed of similar oligosaccharides, but exhibiting different branching patterns, might be detected by the same regulator. For example, laminarin from brown algae and chrysolaminarin from diatoms are composed of $\beta(1\rightarrow3)$ linked glucose units with irregular $\beta(1\rightarrow6)$ branches. Hence, physiological studies with reasonable polysaccharides coupled with proteomics will widen our biochemical knowledge of the substrate specificity of these enzymes and transporters, and thus of polysaccharide decomposition in pelagic and coastal marine environments.

In *Chapter 4* I described the first results on polysaccharide utilization of *Polaribacter* and *Formosa* strains on a limited set of commercially available

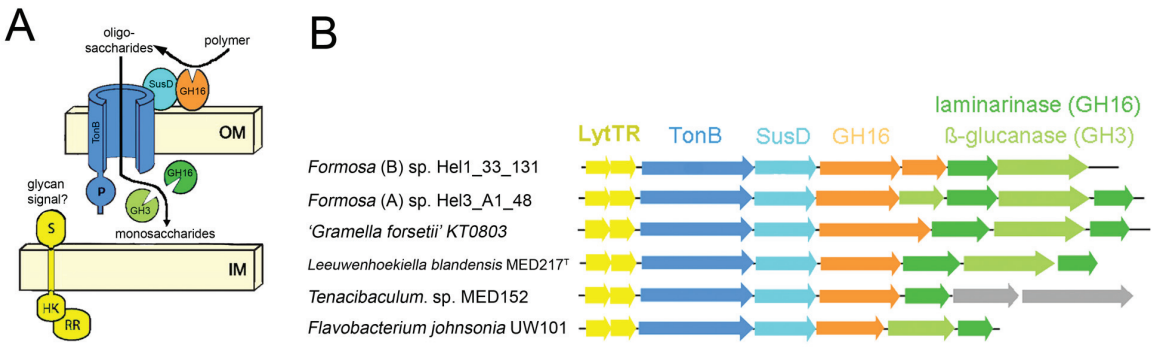

Figure 5.4 Proteins of a polysaccharide utilization loci (PUL) (A) modified after (McBride et al., 2009) and (B) synteny between the laminarin degradation PUL in '*Formosa flavarachnoidea*' (*Formosa* sp. Hel3_A1_48), '*Formosa forsetii*' (*Formosa* sp. Hel1_33_131) and PULs in other *Flavobacteria*. OM, outer membrane; IM, inner membrane; GH, glycoside hydrolases; TonB, TonB-dep. transporter; LytTR, sensor-regulator system.

polysaccharides. This list can be extended in further studies. Currently, Peng Xing (MPI Bremen, Germany) and Frank Unfried (Ernst Moritz Arndt University, Greifswald, Germany) are cultivating '*Polaribacter forsetii*' and '*Formosa forsetii*' on laminarin to identify with proteomics and transcriptomics potential laminarin specific PULs. Co-cultivation in chemostats and batch cultures with polysaccharides or mixtures of polysaccharides could be used to study competition on polysaccharide degradation among the *Polaribacter* and *Formosa* strains. All *Polaribacter* and *Formosa* strains were able to grow on laminarin, but PULs that were predicted to encode for laminarin decomposition are different among the species (Fig. 5.4). Because enzymes and proteins of PULs are localized at the outer membrane or in the periplasm (Fig. 1.5 in *Chapter 1* on page 28), the analysis of sub-proteomes (outer membrane, inner membrane, periplasma) could identify the cellular localization.

An interesting aspect that needs further investigation is linked to the small genomes of *Polaribacter*, *Formosa*, and *Reinekea* strains. So far, missing metabolic pathways and transporters as well as auxotrophy for vitamins,

organic nitrogen, and sulfur sources have been reported for other genome streamlined bacteria. Thus, it is important to understand whether such metabolic consequences apply to the *Polaribacter*, *Formosa*, and *Reinekea* strains or if other features have evolved. First of all, the essential amino acids need to be explored to exclude an excess of amino acids in the medium as peptone and casamino acids, and thus reduce the amount of ammonium that is secreted into the medium.

In conclusion, the isolates obtained in this thesis open many opportunities for further studies on the physiology and genetic potential of coastal marine bacteria, especially of *Flavobacteriaceae*.

References

Alain, K. and Querellou, J. (2009). Cultivating the uncultured: limits, advances and future challenges. *Extremophiles* **13**, 583–594.

Almagro-Moreno, S. and Boyd, E. F. (2009). Insights into the evolution of sialic acid catabolism among bacteria. *BMC Evol Biol* **9**, 118.

Alonso, C., Warnecke, F., Amann, R. and Pernthaler, J. (2007). High local and global diversity of *Flavobacteria* in marine plankton. *Environ Microbiol* **9**, 1253–1266.

Amann, R. I., Ludwig, W. and Schleifer, K. H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**, 143–169.

Bauer, M., Kube, M., Teeling, H., Richter, M., Lombardot, T., Allers, E., Würdemann, C. A., Quast, C., Kuhl, H., Knaust, F. et al. (2006). Whole genome analysis of the marine bacteroidetes '*Gramella forsetii*' reveals adaptations to degradation of polymeric organic matter. *Environ Microbiol* **8**, 2201–2213.

Béjà, O., Aravind, L., Koonin, E. V., Suzuki, M. T., Hadd, A., Nguyen, L. P., Jovanovich, S., Gates, C. M., Feldman, R. A., Spudich, J. L. et al. (2000). Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289**, 1902–1906.

Béjà, O., Koonin, E. V., Aravind, L., Taylor, L. T., Seitz, H., Stein, J. L., Bensen, D. C., Feldman, R. A., Swanson, R. V. and DeLong, E. F. (2002). Comparative genomic analysis of archaeal genotypic variants in a single population and in two different oceanic provinces. *Appl Environ Microbiol* **68**, 335–345.

Béjà, O., Spudich, E. N., Spudich, J. L., Leclerc, M. and DeLong, E. F. (2001). Proteorhodopsin phototrophy in the ocean. *Nature* **411**, 786–789.

Bere, R. (1933). Numbers of bacteria in inland lakes of Wisconsin as shown by the direct microscopic method. *Int Rev Hydobiolo* **29**, 248–263.

Bernardet, J.-F. (2010). *Bergey's Manual of Systematic Bacteriology. The Bacteroidetes, Spirochaetes, Tenericutes (Mollicutes), Acidobacteria, Fibrobacteres, Fusobacteria, Dictyoglomi, Gemmatimonadetes, Lentisphaerae, Verrucomicrobia, Chlamydiae, and Planctomycetes Vol 4*, chapter Class II. *Flavobacteriia* class. nov. Krieg, N.R., Staley, J.T., Brown, D.R., Hedlund, B.P., Paster, B.J., Ward, N.L. et al. (eds), pp. 106–314. Springer, New York.

Bernardet, J. F., Nakagawa, Y., and Holmes, B. (2002). Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* **52**, 1049–1070.

Boraston, A. B., Bolam, D. N., Gilbert, H. J. and Davies, G. J. (2004). Carbohydrate-binding modules: fine-tuning polysaccharide recognition. *Biochem J* **382**, 769–781.

Bruns, A., Nübel, U., Cypionka, H. and Overmann, J. (2003). Effect of signal compounds and incubation conditions on the culturability of freshwater bacterioplankton. *Appl Environ Microbiol* **69**, 1980–1989.

Button, D. K. (1991). Biochemical basis for whole-cell uptake kinetics: specific affinity, oligotrophic capacity, and the meaning of the Michaelis constant. *Appl Environ Microbiol* **57**, 2033–2038.

Button, D. K., Schut, F., Quang, P., Martin, R. and Robertson,

B. R. (1993). Viability and isolation of marine bacteria by dilution culture - theory, procedures, and initial results. *Appl Environ Microbiol* **59**, 881–891.

Campbell, B. J., Waidner, L. A., Cottrell, M. T. and Kirchman, D. L. (2008). Abundant proteorhodopsin genes in the North Atlantic Ocean. *Environ Microbiol* **10**, 99–109.

Cantarel, B., Coutinho, P., Rancurel, C., Bernard, T., Lombard, V. and Henrissat, B. (2009). The Carbohydrate-Active enZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* **37**, D233–D238.

Carini, P., Steindler, L., Beszteri, S. and Giovannoni, S. J. (2012). Nutrient requirements for growth of the extreme oligotroph '*Candidatus* Pelagibacter ubique' HTCC1062 on a defined medium. *ISME J* **7**, 592–602.

Cottrell, M. T. and Kirchman, D. L. (2000). Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. *Appl Environ Microbiol* **66**, 1692–1697.

de la Torre, J. R., Christianson, L. M., Béjà, O., Suzuki, M. T., Karl, D. M., Heidelberg, J. and DeLong, E. F. (2003). Proteorhodopsin genes are distributed among divergent marine bacterial taxa. *Proc Natl Acad Sci USA* **100**, 12830–12835.

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., Clardy, J. and Lewis, K. (2010). Siderophores from neighboring organisms promote the growth of uncultured bacteria. *Chem Biol* **17**, 254–264.

Dufresne, A., Garczarek, L. and Partensky, F. (2005). Accelerated evolution associated with genome reduction in a free-living prokaryote. *Genome Biol* **6**, R14.

Dufresne, A., Salanoubat, M., Partensky, F., Artiguenave, F., Axmann, I. M., Barbe, V., Duprat, S., Galperin, M. Y., Koonin, E. V., Le Gall, F. et al. (2003). Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. *Proc Natl Acad Sci USA* **100**, 10020–10025.

Dupont, C. L., Moffett, J. W., Bidigare, R. R. and Ahner, B. A. (2006). Distributions of dissolved and particulate biogenic thiols in the subartic Pacific Ocean. *Deep Sea Res Pt I* **53**, 1961–1974.

Dupont, C. L., Rusch, D. B., Yooseph, S., Lombardo, M. J., Richter, R. A., Valas, R., Novotny, M., Yee-Greenbaum, J., Selengut, J. D., Haft, D. H. et al. (2012). Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. *ISME J* **6**, 1186–1199.

Eilers, H. (2000). *Pelagic microbial communities in the North Sea: cultivation, diversity and in situ dynamics.* Ph.D. thesis, University of Bremen, Bremen, Germany.

Eilers, H., Pernthaler, J. and Amann, R. (2000a). Succession of pelagic marine bacteria during enrichment: a close look at cultivation-induced shifts. *Appl Environ Microbiol* **66**, 4634–4640.

Eilers, H., Pernthaler, J., Glöckner, F. O. and Amann, R. (2000b). Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Appl Environ Microbiol* **66**, 3044–3051.

Eilers, H., Pernthaler, J., Peplies, J., Glöckner, F. O., Gerdts,

G. and Amann, R. (2001). Isolation of novel pelagic bacteria from the German Bight and their seasonal contributions to surface picoplankton. *Appl Environ Microbiol* **67**, 5134–5142.

Esko, J. D., Kimata, K. and Lindahl, U. (2009). *Essentials of Glycobiology*, chapter 16: Proteoglycans and sulfated glycosaminoglycans. Varki A., Cummings R.D., Esko J.D., Freeze H.H., Stanley P., Bertozzi C.R., Hart G.W. and Etzler M.E. (eds). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fernández-Gómez, B., Richter, M., Schüler, M., Pinhassi, J., Acinas, S., González, J., J. M. and Pedrós-Alió, C. (2013). Ecology of marine Bacteroidetes: a comparative genomics approach. *ISME J* **7**, 1026–1037.

Flärdh, K., Cohen, P. S. and Kjelleberg, S. (1992). Ribosomes exist in large excess over the apparent demand for protein synthesis during carbon starvation in marine *Vibrio* sp. strain CCUG 15956. *J Bacteriol* **174**, 6780–6788.

Giebel, H. A., Kalhoefer, D., Lemke, A., Thole, S., Gahl-Janssen, R., Simon, M. and Brinkhoff, T. (2011). Distribution of *Roseobacter* RCA and SAR11 lineages in the North Sea and characteristics of an abundant RCA isolate. *ISME J* **5**, 8–19.

Giovannoni, S. J., Tripp, H. J., Givan, S., Podar, M., Vergin, K. L., Baptista, D., Bibbs, L., Eads, J., Richardson, T. H., Noordewier, M. et al. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science* **309**, 1242–1245.

Gobler, C. J., Norman, C., Panzeca, C., Taylor, G. T. and Sañudo-Wilhelmy, S. A. (2007). Effect of B-vitamins (B_1, B_{12}) and in-

organic nutrients on algal bloom dynamics in a coastal ecosystem. *Aquat Microb Ecol* **49**, 181–194.

Gómez-Consarnau, L., Akram, N., Lindell, K., Pedersen, A., Neutze, R., Milton, D. L., González, J. M. and Pinhassi, J. (2010). Proteorhodopsin phototrophy promotes survival of marine bacteria during starvation. *PLoS Biol* **8**, e1000358.

Gómez-Consarnau, L., González, J. M., Coll-Lladó, M., Gourdon, P., Pascher, T., Neutze, R., Pedrós-Alió, C. and Pinhassi, J. (2007). Light stimulates growth of proteorhodopsin-containing marine Flavobacteria. *Nature* **445**, 210–213.

Gómez-Pereira, P. R., Fuchs, B. M., Alonso, C., Oliver, M. J., van Beusekom, J. E. E. and Amann, R. (2010). Distinct flavobacterial communities in contrasting water masses of the North Atlantic Ocean. *ISME J* **4**, 472–487.

Gómez-Pereira, P. R., Schüler, M., Fuchs, B. M., Bennke, C., Teeling, H., Waldmann, J., Richter, M., Barbe, V., Bataille, E., Glöckner, F. O. et al. (2012). Genomic content of uncultured *Bacteroidetes* from contrasting oceanic provinces in the North Atlantic Ocean. *Environ Microbiol* **14**, 52–66.

González, J. M., Fernández-Gómez, B., Fernàndez-Guerra, A., Gómez-Consarnau, L., Sánchez, O., Coll-Lladó, M., del Campo, J., Escudero, L., Rodríguez-Martínez, R., Alonso-Sáez, L. et al. (2008). Genome analysis of the proteorhodopsin-containing marine bacterium *Polaribacter* sp. MED152 (Flavobacteria). *Proc Natl Acad Sci USA* **105**, 8724–8729.

Grady, J. K., Chasteen, N. D. and Harris, D. C. (1988). Radicals from 'Goods' buffers. *Anal Biochem* **173**, 111–115.

Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **208**, 309–316.

Jannasch, H. and Mateles, R. I. (1974). Experimental bacterial ecology studied in continuous culture. *Adv Microb Physiol* **11**, 165–212.

Jannasch, H. W. and Jones, G. E. (1959). Bacterial populations in sea water as determined by different methods of enumeration. *Limnol Oceanogr* **4**, 128–139.

Kaprelyants, A. S., Gottschal, J. C. and Kell, D. B. (1993). Dormancy in non-sporulating bacteria. *FEMS Microbiol Rev* **104**, 271–286.

Kirchman, D. L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**, 91–100.

Kirchman, D. L., Yu, L. Y. and Cottrell, M. T. (2003). Diversity and abundance of uncultured *Cytophaga*-like bacteria in the Delaware Estuary. *Appl Environ Microbiol* **69**, 6587–6596.

Konstantinidis, K. T. and DeLong, E. F. (2008). Genomic patterns of recombination, clonal divergence and environment in marine microbial populations. *ISME J* **2**, 1052–1065.

Konstantinidis, K. T. and Tiedje, J. M. (2005). Genomic insights that advance the species definition for prokaryotes. *Proc Nat Acad Sci USA* **102**, 2567–2572.

Krieg, N. and Hoffman, P. S. (1986). Microaerophily and oxygen toxicity. *Annu Rev Microbiol* **40**, 107–130.

Lage, O. M., Vasconcelos, M., Soares, H., Osswald, J. M., Sansonetty, F., Parente, A. M. and Salema, R. (1996). Suitability of the pH buffers 3-[*N-N-bis*(hydroxethyl)amino]-2-hydroxypropanesulfonic

acid and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid for *in vitro* copper toxicity studies. *Arch Environ Contam Toxicol* **31**, 199–205.

Laine, R. A. (1994). A calculation of all possible oligosaccharide isomers both branched and linear yields 1.05×10^{12} structures for a reducing hexasaccharide: the *Isomer Barrier* to development of single-method saccharide sequencing or synthesis systems. *Glycobiology* **4**, 759–767.

Lee, C., Wakeham, S. and Arnosti, C. (2004). Particulate organic matter in the sea: the composition conundrum. *Ambio* **33**, 565–575.

Man, D. L., Wang, W. W., Sabehi, G., Aravind, L., Post, A. F., Massana, R., Spudich, E. N., Spudich, J. L. and Béjà, O. (2003). Diversification and spectral tuning in marine proteorhodopsins. *EMBO J* **22**, 1725–1731.

Martens, E. C., Lowe, E. C., Chiang, H., Pudlo, N. A., Wu, M., McNulty, N. P., Abbott, D. W., Henrissat, B., Gilbert, H. J., Bolam, D. N. et al. (2011). Recognition and degradation of plant cell wall polysaccharides by two human gut symbionts. *PLoS Biol* **9**, e1001221.

McBride, M. J., Xie, G., Martens, E. C., Lapidus, A., Henrissat, B., Rhodes, R. G., Goltsman, E., Wang, W., Xu, J., Hunnicutt, D. W. et al. (2009). Novel features of the polysaccharide-digesting gliding bacterium *Flavobacterium johnsoniae* as revealed by genome sequence analysis. *Appl Environ Microbiol* **75**, 6864–6875.

Moore, E. R. B. and Rosselló-Móra, R. (2011). MALDI-TOF MS: A return to phenotyping in microbial identification? *Syst Appl Microbiol* **34**, 1.

Morris, J. J., Johnson, Z. I., Szul, M. J., Keller, M. and Zinser, E. R. (2011). Dependence of the cyanobacterium *Prochlorococcus* on

hydrogen peroxide scavenging microbes for growth at the ocean's surface. *Plos One* **6**, 13.

Morris, J. J., Lenski, R. E. and Zinser, E. R. (2012). The Black Queen Hypothesis: evolution of dependencies through adaptive gene loss. *mBio* **3**, e00036–12.

Oesterhelt, D. and Stoeckenius, W. (1973). Functions of a new photoreceptor membrane. *Proc Natl Acad Sci USA* **70**, 2853–2857.

Pearl, S., Gabay, C., Kishony, R., Oppenheim, A. and Balaban, N. Q. (2008). Nongenetic individuality in the host-phage interaction. *PLoS Biol* **6**, 957–964.

Pedersen, H. L., Fangel, J. U., McCleary, B., Ruzanski, C., Rydahl, M. G., Ralet, M.-C., Farkas, V., von Schantz, L., Marcos, S. E., Andersen, M. C. F. et al. (2012). Versatile high-resolution oligosaccharide microarrays for plant glycobiology and cell wall research. *J Biol Chem* **287**, 39429–39438.

Rappé, M. S., Connon, S. A., Vergin, K. L. and Giovannoni, S. J. (2002). Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature* **418**, 630–633.

Reisch, C. R., Moran, M. A. and Whitman, W. B. (2011). Bacterial catabolism of dimethylsulfoniopropionate (DMSP). *Front Microbiol* **2**, 172.

Riedel, T., Tomasch, J., Buchholz, I., Jacobs, J., Kollenberg, M., Gerdts, G., Wichels, A., Brinkhoff, T., Cypionka, H. and Wagner-Döbler, I. (2010). Constitutive expression of the proteorhodopsin gene by a flavobacterium strain representative of the

proteorhodopsin-producing microbial community in the North Sea. *Appl Environ Microbiol* **76**, 3187–3197.

Rosselló-Mora, R. and Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.

Roy, R., Katzenellenbogen, E. and Jennings, H. J. (1984). Improved procedures for the conjugation of oligosaccharides to protein by reductive amination. *Can J Biochem Cell B* **62**, 270–275.

Rye, P. D. (1996). Sweet and sticky: carbohydrate coated magnetic beads. *Nature Biotechnol* **14**, 155–157.

Rye, P. D. and Bovin, N. V. (1997). Selection of carbohydrate-binding cell phenotypes using oligosaccharide-coated magnetic particles. *Glycobiology* **7**, 179–182.

Sabehi, G., Loy, A., Jung, K. H., Partha, R., Spudich, J. L., Isaacson, T., Hirschberg, J., Wagner, M. and Béjà, O. (2005). New insights into metabolic properties of marine bacteria encoding proteorhodopsins. *PLoS Biol* **3**, 1409–1417.

Sañudo-Wilhelmy, S. A., Cutter, L. S., Durazo, R., Smail, E. A., Gómez-Consarnau, L., Webb, E. A., Prokopenko, M. G., Berelson, W. M. and Karl, D. M. (2012). Multiple B-vitamin depletion in large areas of the coastal ocean. *Proc Natl Acad Sci USA* **109**, 14041–14045.

Seljelid, R., Bøgwald, J., Rasmussen, L. T., Larm, O., Hoffman, J., Berge, A. and Ugelstad, J. (1985). *In vivo* activation of mouse macrophages with β-l,3-D-glucan-derivatized plastic beads. *Scand J Immunol* **21**, 601–605.

Severi, E., Hood, D. W. and Thomas, G. H. (2007). Sialic acid utilization by bacterial pathogens. *Microbiol* **153**, 2817–2822.

Stackebrandt, E. and Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiology Today* **33**, 152–155.

Sternemarr, R., Blevitt, J. M. and Gerace, L. (1992). O-linked glycoproteins of the nuclear pore complex interact with a cytosolic factor required for nuclear protein import. *J Cell Biol* **116**, 271–280.

Stevens, H., Simon, M. and Brinkhoff, T. (2009). Cultivable bacteria from bulk water, aggregates, and surface sediments of a tidal flat ecosystem. *Ocean Dynam* **59**, 291–304.

Suzuki, M. T., Rappé, M. S., Haimberger, Z. W., Winfield, H., Adair, N., Ströbel, J. and Giovannoni, S. J. (1997). Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl Environ Microbiol* **63**, 983–989.

Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J. et al. (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336**, 608–611.

Thingstad, F. and Billen, G. (1994). Microbial degradation of *Phaeocystis* material in the water column. *J Mar Syst* **5**, 55–65.

Urbani, R., Magaletti, E., Sist, P. and Cicero, A. M. (2005). Extracellular carbohydrates released by the marine diatoms *Cylindrotheca closterium*, *Thalassiosira pseudonana* and *Skeletonema costatum*: effect of P-depletion and growth status. *Sci Total Environ* **353**, 300–306.

Venter, J. C., Remington, K., Heidelberg, J. F., Halpern, A. L., Rusch, D., Eisen, J. A., Wu, D. Y., Paulsen, I., Nelson, K. E., Nelson, W. et al. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**, 66–74.

Vimr, K. A., E. R.and Kalivoda, Deszo, E. L. and Steenbergen, S. M. (2004). Diversity of microbial sialic acid metabolism. *Microbiol Mol Biol Rev* **68**, 132–153.

Wakeham, S. G., Lee, C., Hedges, J. I., Hernes, P. J. and Peterson, M. L. (1997). Molecular indicators of diagenetic status in marine organic matter. *Geochim Cosmochim Acta* **61**, 5363–5369.

Wang, D., He, J., Rosenzweig, N. and Rosenzweig, Z. (2004). Superparamagnetic Fe_2O_3 beads–CdSe/ZnS quantum dots core–shell nanocomposite particles for cell separation. *Nano Lett* **4**, 409–413.

Warren, R. A. J. (1996). Microbial hydrolysis of polysaccharides. *Annu Rev Microbiol* **50**, 183–212.

Weinbauer, M. G., Brettar, I. and Höfle, M. G. (2003). Lysogeny and virus-induced mortality of bacterioplankton in surface, deep, and anoxic marine waters. *Limnol Oceanogr* **48**, 1457–1465.

Weinbauer, M. G. and Suttle, C. A. (1999). Lysogeny and prophage induction in coastal and offshore bacterial communities. *Aquat Microb Ecol* **18**, 217–225.

Winkelmann, N. and Harder, J. (2009). An improved isolation method for attached-living *Planctomycetes* of the genus *Rhodopirellula*. *J Microbiol Meth* **77**, 276–284.

Withers, S. (accessed Dez. 2012). Glycoside Hydrolase family 2. *CAZypedia* **available at http://www.cazypedia.org/**.

Yoshizawa, S., Kawanabe, A., Ito, H., Kandori, H. and Kogure, K. (2012). Diversity and functional analysis of proteorhodopsin in marine *Flavobacteria*. *Environ Microbiol* **14**, 1240–1248.

Zengler, K., Toledo, G., Rappé, M., Elkins, J., Mathur, E. J., Short, J. M. and Keller, M. (2002). Cultivating the uncultured. *Proc Natl Acad Sci USA* **99**, 15681–15686.

Zigler, J. S. J. ., Lepe-Zuniga, J., Vistica, B. and Gery, I. (1985). Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium. *In Vitro Cell Dev Biol* **21**, 282–287.

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Appendix – Variations of pelagic bacterial communities in the North Atlantic Ocean coincide with water bodies

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Contributions to the manuscript:
R.L.H. and J.H. designed research and project outline. C.P. performed sampling on the VISION cruise and did T-RFLP. R.L.H. did statistical analysis and the in silico T-RFLP. R.L.H., B.M.F. and J.H. conceived, wrote and edited the manuscript.

Chapter is *in review* for *Aquatic Microbiology Ecology*

Abstract

Biological and pysico-chemical characteristics define ecological provinces. On a transect along the 30°W meridian from 67°N to 34°N, the North Atlantic Ocean was partitioned into four ecological provinces and nine water masses. Whether this ecological provinces were reflected in distinct bacterial populations was studied by terminal restriction fragment length polymorphism (T-RFLP) analysis of bacterial 16S rRNA genes present in water samples along the transect and at depths between sea-surface and 500 meters. *Synechococcus* prevailed in the North whereas *Prochlorococcus* was more abundant in southern sampling stations. Microbial communities were generally more diverse in phototrophic layers above the pycnocline. Distinct communities were detected in the epipelagic along the latitudinal transect through the different water masses, with a second major diversity change from the epipelagic to the mesopelagic zone. Differences in T-RFLP patterns coincided well with differences in the physico-chemical conditions of the sampling sites. *In silico* analyzes were developed to assign phylogenetic groups to terminal restriction fragments (TRFs) and detected for instance populations of high-light and low-light ecotypes of *Prochlorococcus*. Water masses in the North Atlantic Ocean hosted different bacterial communities, including individual populations that may serve as biological marker for the water mass.

Introduction

The open ocean harbors a diversity of microorganisms which have often a regional distribution. One example are unicellular cyanobacteria affiliating to *Synechococcus* and *Prochlorococcus* (Li, 1994; Liu et al., 1997; Veldhuis et al., 1997). The variety of habitats results from annual seasonal changes, intense atmospheric events, the thermohaline circulation and currents throughout the ocean (Platt and Sathyendranath, 1999; Teeling et al., 2012). Longhurst partitioned the ocean based on physical forcing into 56 ecological provinces and provided static definitions of the province boundaries (Longhurst et al., 1995). These ecological provinces are regions or water masses defined by physico-chemical (e.g. temperature, salinity, bathymetry) and biological (e.g. chl *a* concentration, vertical distribution of bacterioplankton) characteristics, and a common history (Emery and Meincke, 1986; Devred et al., 2007). Because ocean surface color significantly correlates with water column integrated chlorophyll concentrations, photic depth, and nutrient fields, ecological provinces can be discriminated by the global time series of satellite ocean color and sea surface temperature (Esaias et al., 2000; Oliver and Irwin, 2008). The North Atlantic Current and its prolongation, the North Atlantic Drift Current, divides the North Atlantic Ocean into a northern and a southern part. Two branches extend at 38°N 44°W northeastward along the continental slope and southeastward along the continental slope feeding the current around the North Atlantic Gyre (Mann, 1967). Along the 30°W meridian from 67°N to 34°N, the North Atlantic Ocean contains nine water masses in four Longhurstian provinces (suppl. Fig. A.S1): one Boreal Polar (BPLR), four Atlantic Arctic (ARCT), two North Atlantic Drift (NADR) and two North Atlantic Subtropical Gyre (NAST) (Longhurst et al., 1995; Gómez-Pereira et al., 2010). Physical (temperature, salinity), chemical (nutrients) and biological data (chlorophyll

a, picoplankton, nanophytoplankton, enzyme activities) clearly indicated gradients along the transect, from cold and nutrient rich water masses in the North to warm oligotrophic water masses in the South (Gómez-Pereira et al., 2010; Schattenhofer et al., 2011; Arnosti et al., 2012). Coincidence of ecological provinces in surface water the North Atlantic Ocean and local bacterioplankton populations was recently shown for *Flavobacteria* clades (Gómez-Pereira et al., 2010) and picoplanktonic populations (Schattenhofer et al., 2011). So far, a characterization of the diversity of the bacterioplankton within water masses and with depth is missing, in the North Atlantic Ocean. We hypothesized a strong correlation for all bacterial clades with water masses, and conducted a cruise from Island (66° 39.27'N) to the Azores (66° 39.27'N) along a latitudinal gradient 30°W, thus north of cruises of the Atlantic meridional transect program (Aiken et al., 2000). Epipelagic and mesopelagic bacterial communities were investigated by terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA gene amplicons and flow cytometry counting of *Prochlorococcus* and *Synechococcus* populations.

Material and methods

Sampling

Water samples were obtained on the Maria S. Merian during the VISION (diVersIty, Structure, functION) cruise MSM03/1 (September 2006) with a CTD rosette equipped with 24 Niskin bottles (suppl. Fig. A.S1). At each depth seawater aliquots were sampled in triplicate: the biomass of a 200 ml aliquot was concentrated on a 0.2 μm Isopore filter with a diameter of 45 mm (Millipore, Billerica, MA) and the filter was frozen immediately and stored at –20 ℃. Salinity, temperature, the concentrations of phosphate, ammonium, nitrite and nitrate were taken from (Gómez-Pereira et al., 2010). The cell numbers of *Synechococcus*, *Prochlorococcus* and of the total

bacterioplankton were determined by flow cytometric analyses as described in (Tarran et al., 2006).

DNA extraction and T-RFLP

From each station depth, three biological replicates were analyzed. Genomic DNA was isolated from half a filter, representing 100 ml water sample, based on a protocol of (Boström et al., 2004). The filter half was placed in a 2.2 ml sample vial and extracted with 525 μL lysis buffer and 11 μL lysozyme (50 mg ml^{-1}) for 30 min at 37 ℃ in an overhead shaker. After addition of 60 μL 10% SDS and 3 μL proteinase K (20 mg ml^{-1}), the extraction was continued for 12 h at 55 ℃ in the overhead shaker. The supernatant was transferred and incubated together with 100 μL isopropanol for 1 h at room temperature. The DNA was precipitated with 15000x g for 30 min at 4 ℃. The pellet was washed with 100 μL cold ethanol, precipitated a second time and air dried. The DNA was dissolved in 50 μL water and quantification yielded 10 to 50 ng genomic DNA per sample. Amplification of the partial 16S rRNA gene was performed with the fluorescently labeled primers 27F (FAM, 5'-AGA GTT TGA TYM TGG CTC AG-3') and 907R (HEX, 5'-CCG TCA ATT CCT TTR AGT TT-3'), targeting all bacteria (Muyzer et al., 1995). The PCR reaction contained 12.5 μL PCR Master Mix (Promega GmbH, Mannheim, Germany), 4 μM of forward and of reverse primer, and 1–5 ng DNA template in 25 μL. The cycle program was 95 ℃ for 1 min, 33 cycles of 95 ℃ for 1 min, 60 ℃ for 1 min and 72 ℃ for 3 min, followed by 60 ℃ for 60 min. PCR amplicons were purified on Sephadex columns (Sephadex™G-50 Superfine, Amersham Biosciences AB, Uppsala, Sweden). Approximately 25 ng of PCR amplicon were digested in a total volume of 10 μL using 5 U of the restriction enzyme *AluI* (Fermentas, Burlington, Canada) at 37 ℃ for 3 hours, followed by heat inactivation at 65 ℃ for 30 min. After purification on Sephadex columns,

terminal restriction fragments (TRFs) were detected on an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems, California) equipped with an 80 cm capillary, a POP-7 polymer and the filter set D (Filter DS-30). The ROX-labeled MapMarker® 1000 (Eurogentec, Belgium) served as a size standard between 50 bp and 1000 bp.

TRF pattern analyses

T-RFLP patterns were inspected manually with the software Genetic Analyser 3.7 (Applied Biosystems, California, USA). The fluorescence intensity threshold was set to 20 units and the fragments with a size between 50 and 1000 nucleotides were identified and sized (Local Southern, normalization within each run, sum of signals) with the internal size marker. For comparative analyses, the individual pattern were processed applying the interactive binner (Ramette, 2009). The binning size was 1 nucleotide and the binning shift 0.5 nucleotides. Due to a naming of each TRF by its start of the binning window we added 0.5 bases to the TRF length in naming TRFs. The resulting pattern with normalized peak areas (RFI, relative fluorescence intensity of 100% corresponds to the sum of peak areas in each T-RFLP profile) were visualized in rank versus cumulated abundance curves with the k-dominance plot in PRIMER-E (v.6, PRIMER-E, Plymouth Marine Laboratory, UK) (Clarke, 1993). Inspection by Genetic Analyser and the k-dominance plots served to remove outliers within the triplicates and identify the final T-RFLP data set (suppl. Fig. A.S2). The constrained (canonical) correspondence (Ter Braak, 1986) analysis was used to relate the compositional variation in the bacterial community of the sampling sites as χ^2 (chi-squared) distances to the observed environmental variation by canonical correlations, and perform a weighted linear mapping, without information of depth and longitude. For comparability a sampling site-similarity matrix was generated using the Bray-Curtis coefficient by

comparing the RFI of each TRF with regard to every pair wise combination of all stations and depth, with 999 permutations. Non-parametric multivariate statistical analysis was performed using PRIMER-E and the R package VEGAN (v.1.8-3 Dixon, 2003). Visual comparisons between bacterial communities of predefined oceanic provinces (BPLR, ARCT, NADR, NAST) were explored by ordination using non-metric multidimensional scaling (nMDS), with 100 random restarts and 999 iterations. As third method we used the hierarchical clustering to group the sampling sites. Visualization was performed by adding the information of the hierarchical tree into the nMDS plot. A consistent biplot was obtained in a fitting of the environmental conditions into the nMDS plot applying the function envfit of the R package VEGAN with 1000 permutation and p-values smaller than 0.001, but without information of depth and longitude (Dixon, 2003). Analysis of similarity (ANOSIM) in PRIMER-E was used to verify the significance of water mass specific clustering of bacterial communities by testing the null hypothesis that bacterial communities from the same water mass were more similar to each other than to bacterial communities in different water masses. To test for differences in bacterial communities between water masses in the epipelagic zone and differences in epipelagic and mesopelagic bacterial communities two-way crossed ANOSIM statistics were generated. ANOSIM statistics were based on the same sampling site-similarity matrix of Bray-Curtis coefficients, as for nMDS, and computed with 999 permutations. To identify which TRF formed a strong gradient along the latitude and into the depth we used the principal components analysis (PCA), transforming the variable space (RFI of each TRF of each sampling site) into its orthogonal principal components. Afterward, the eigenvectors of the TRF and principal components scores of the sampling sites were visualized. Similarity percentage analysis (SIMPER) was used to get the significance of TRFs in water masses. A significant TRF was

defined as one with (i) an average RFI within the represented water mass or water masses of at least double as high as in the other water masses, (ii) the ratio higher than one between the contribution to the average Bray-Curtis dissimilarity (Average Dissimilarity) between all pairs of sampling sites (one within the represented oceanic provinces and one outside), and the standard deviation (SD) of those contributions, (iii) and a RFI of more than 4% in at least one sampling site. Corresponding peak of the significant representative TRF were again inspected manually in the original T-RFLP pattern with Genetic Analysis 3.7, to confirm that the analyzed fluorescent signal was unaffected by neighboring TRF. Finally, the biogeography of the TRFs were visualized in Ocean Data View (v3.4.2, ODV, AWI, Bremerhaven, Germany) (Schlitzer, 2002).

In silico prediction of the fragment size

16S rRNA gene sequences were retrieved with the ARB program (Ludwig et al., 2004) from the SILVA database (rel102ref, 391167 bacterial sequences) (Pruesse et al., 2007) by targeting both T-RFLP primers with 0 to 2 mismatches. Four sets of sequences were generated, (i) 135761 sequences of all phyla, (ii) 87 out of 361 sequences of *Synechococcus*, (iii) 382 out of 944 sequences of *Prochlorococcus* and (iv) 233 out of 885 sequences of a *Bacteroidetes* specific clone library retrieved from Gómez-Pereira et al. (2010). These sequences were trimmed to the T-RFLP amplicon size. The program TRFragCalc (m-file is available at http://www.mpi-bremen.de_Richard_Hahnke.html) written in MATLAB (v.2.9.0.529 R2009b, MATLAB The Language of Technical Computing, The MathWorks, Natick, USA) was applied to import sequences, to identify the restriction recognition site, and to calculate the resulting T-RFLP fragments. Starting with an *in silico* fragment, e.g. iTRF_128nt for *Synechococcus*, we investigated the distribution of TRF in a range of $\pm$ 5 nucleotides,

e.g. TRF_123nt to TRF_133nt. This window of 10 nucleotides is necessary because an absolute determination of the length of TRFs with capillary electrophoresis is currently not possible (Bruland et al., 1999; Hahn et al., 2001; Olejniczak et al., 2005).

Results

Oceanographic changes and the bacterial diversity determined by T-RFLP
The North Atlantic Ocean at depths between 20 m and 500 m along the 30°W meridian from the productive cold Greenland current (66°39´N) across the cold north and warm south of the North Atlantic Current to the oligotrophic central Atlantic Ocean (34°24´N) (suppl. Fig. A.S1) contained a bacterial diversity revealed in the presence of 467 terminal restriction fragments (TRFs) in all samples (γ diversity). Most samples had 58 to 105 TRFs (25% and 75% quantil, α diversity), median 86 TRFs (suppl. Fig. A.S3). The Shannon diversity index based on the relative abundance of the TRFs was large in the epipelagic zone, with a high diversity north and south of the North Atlantic Drift (Fig. A.1).

T-RFLP pattern and environmental conditions
The fragment pattern of the sampling sites were constrained with environmental conditions (salinity, conductivity, temperature, and the concentration of dissolved oxygen, ammonium and nitrate) in a unimodal model. The constrained (or canonical) correspondence analysis (CCA) covered one third of the total variance (inertia = 2.07), reflected by a mean squared contingency coefficient of the constrained axes with 31% (inertia = 0.63). The CCA revealed a distribution of sampling sites along a latitudinal gradient and with water depth (Fig. A.2).

Oxygen, temperature and salinity were coinciding with the first dimen-

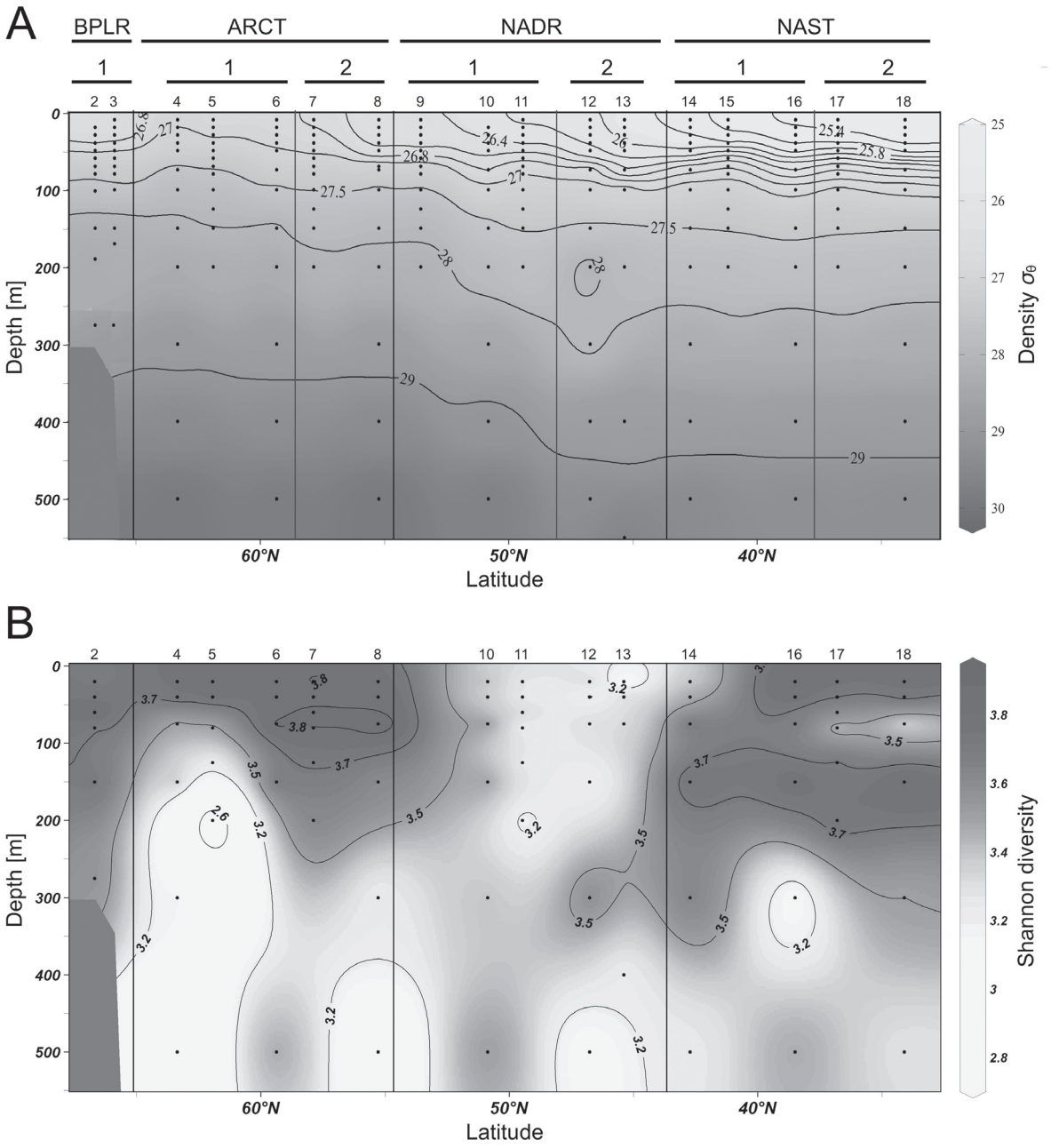

Figure A.1 Density σ_Θ (A) and Shannon diversity index of bacterial 16S rRNA T-RFLP profiles (B) in the North Atlantic Ocean. Water was sampled from the East Greenland Current (BPLR, between Greenland and Iceland) through the areas north (ARCT) and south (NADR) of the Gulf Stream to the North Atlantic Gyre (NAST, south of the Azores).

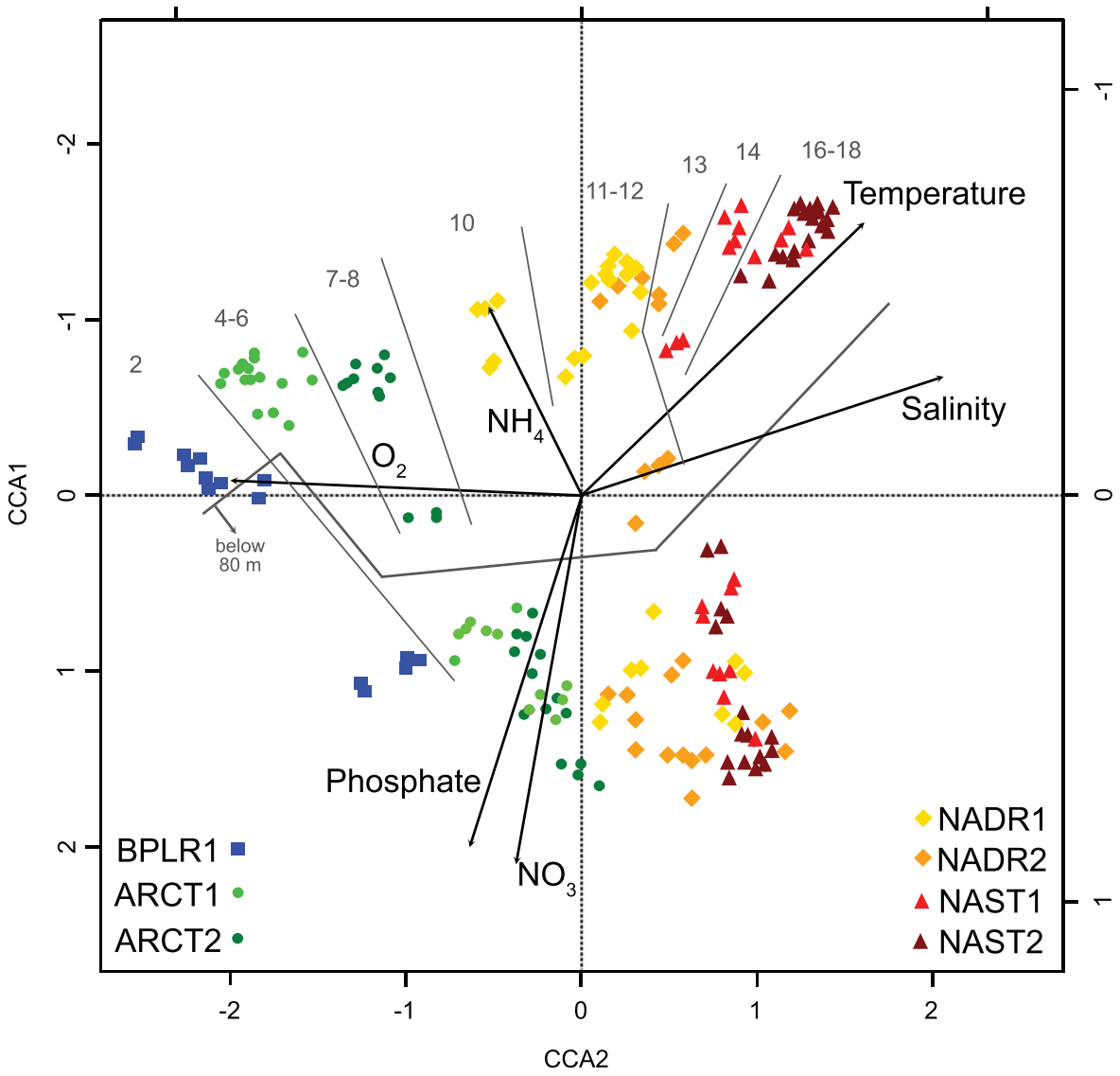

Figure A.2 Canonical correspondence analysis of T-RFLP profiles and constrained environmental parameters. The CCA presentation of the differences in the TRF pattern revealed a clustering of water masses along a latitudinal gradient (BPLR, square; ARCT, dot; NADR, diamond; NAST, triangle) and a separation in either epipelagic or mesopelagic origin (pycnocline at 50 to 100 m depth). The depth gradient is represented by the amount of ammonium, nitrate and phosphate whereas the latitude gradient is represented by the amount of oxygen, temperature and salinity, because including the depth and latitude into the calculation would ultimately change the two dimensional visualization (abundances were analyzed constrained to the environmental data). Numbers represent sampling stations.

sion, a proxi for latitude, whereas ammonium, nitrate and phosphate were proxies for the water depth. Bacterial communities from the surface water were well separated from bacterial communities from water deeper than 80 meter. This coincided with a pycnocline (suppl. Fig. A.S1), and suggested 80 m as border between the mixed layer and stratified deeper waters. The ANOSIM test showed that differences between epipelagic and mesopelagic bacterial communities were significant (global $R = 0.79$, $p < 0.1\%$). A hierarchical clustering at 48% similarity defined three groups of samples: (i) an epipelagic BPLR-ARCT cluster, (ii) an epipelagic NADR-NAST cluster, and (iii) one common cluster of mesopelagic samples (suppl. Fig. A.S4). Samples from the Longhurstian provinces BPLR, ARCT, NADR and NAST formed cluster. The seven smaller clusters of surface water (Fig. A.1) coincided with the water masses defined by Gómez-Pereira et al. (2010). Within the provinces, sampling stations of the water masses BPLR (station 2) , ARCT1 (st. 4–6) and ARCT2 (st. 7–8) were separated. The NADR (stations 10–13) showed a high variation, reflecting the dynamic environment of the ocean current. The geographical distance between stations 10 and 11 is smaller than between stations 11 and 12. In contrast, the dissimilarity of the bacterial community between stations 10 and 11 was large, compared to the dissimilarity between stations 11 and 12 (Fig. A.2). Stations 16 to 18 of the water masses NAST1 and NAST2 could not be distinguished on the basis of the T-RFLP pattern. Station 14 and 16 belonged to the water mass NAST1, but station 16 clustered with stations of the water mass NAST2 and station 14 was separated from station 16. The significant separation of bacterial communities in the BPLR, ARCT1 and ARC2 water masses, compared to the more similar sampling sites in the NADR and NAST province, was reflected in the ANOSIM results. Overall, the differences between bacterial communities of the water masses were significant (global $R = 0.60$, $p < 0.1\%$). Pairwise tests of epipelagic water

masses showed, that the water masses BPLR1 and NAST2, ARCT2 and NADR1, NADR2 and NAST1 were well separated (see ANOSIM results in supplementary.

Water masses within the same province (e.g. NAST1 and NAST2) had a larger shared bacterial community. To confirm the clustering of bacterial populations with water masses along the latitude and an independence from the CCA method (uses χ^2 distances), the similarity between bacterial communities of individual sampling sites was calculated with the Bray-Curtis similarity (based on relative abundances) and the Sörensen index (β diversity, based on presents\absents). The nMDS of both indices revealed a distribution of sampling sites along the four provinces and with water depth (suppl. Fig. A.S4), comparable to the results of the CCA. This supports our hypothesis of a change in bacterial communities with water masses (β diversity). However, the discrimination of bacterial communities in different water masses was more pronounced with relative abundances. Altogether, the applied nonparametric statistical analyses demonstrated the presence of individual bacterial communities in the different water masses.

Characteristic terminal restriction fragments for individual oceanic provinces

Differences between bacterial communities present in water masses were traced to individual TRFs with similarity percentage analysis (SIMPER, Tab. 6.1). Among the abundant TRFs, only TRF_58nt and TRF_152nt were detected in all stations with less than 80 m water depth, whereas forty TRFs varied in their presence. In the north, TRF_203nt and TRF_259nt were characteristic for the BPLR. ARCT1 and contained statistically significant populations of the TRF_125nt and TRF_605nt, ARCT2 the TRF_158nt, TRF_193nt and TRF_201nt, and NADR2 the TRF_158nt. The sampling sites in the NADR province had a high abundance of TRF_195nt, and

in the NAST province of TRF_183nt, TRF_207nt and TRF_242nt. The analysis revealed a number of TRFs significant for two adjacent provinces. TRF_204nt and TRF_217nt were less abundant in the north and in the south, respectively. The TRF_125nt, TRF_189nt, TRF_193nt, TRF_194nt, and TRF_204nt had the highest maximum RFI of 11.9% to 44.1%. Water samples below 80 m were characterized by TRF_152nt and TRF_241nt (Tab. 6.1). The principal component analysis (PCA) was used to identify TRFs forming a strong increasing RFI along the latitude or into the depth (suppl. Tab. A.S5). This first principal component distinguished between the northern (BPLR, ARCT) and the southern provinces (NADR, NAST) and revealed a strong influence of the latitude (55.8% of the total variation). The second principal component covered 13.6% of the total variance and distinguished depths above and below 80 m. The largest eigenvector parallel to the first principal component had the TRF_189nt. The second principal component had major contributions from TRF_125nt, TRF_152nt, TRF_193nt, and TRF_204nt. Thus, the strong regionallity of individual TRFs characterized the bacterial communities of different water masses along the transect in the North Atlantic Ocean (Fig. A.3).

Assignment of terminal restriction fragments to bacterial taxa

With TRFragCalc, written in MATLAB for this study, we assigned *in silico* terminal restriction fragments to cyanobacteria and compared the result with measured TRFs (Fig. 4 1b–3b) and fluorescence-detection of cyanobacteria by flow cytometry (Fig. A.4 1a–3a). *In silico* terminal restriction fragments (named iTRFs) of *Synechococcus* 16S rRNA genes had 73, 128, 190 and 205 nucleotides. The iTRF_128nt originated from 246 sequences of *Synechococcus* clade I and 3 sequences of *Synechococcus* clade III (overall 286 cyanobacteria in 327 sequences). TRF abundance pattern and the by flow cytometry determined distribution of *Synechococcus* cell counts in-

Table 6.1 Abundance and significance of terminal restriction fragments (TRF) that were representative for water masses. Depicted are the RFI maximum and the associated sampling site (station, depth), the average RFI in the water masses (BPLR, ARCT1/2, NADR1/2, NAST1/2), the average RFI of TRFs in the represented oceanic province (in) and in all other provinces (out), mean dissimilarity (Diss/SD) from SIMPER. Additionally, the same information is given for the sum of TRF (sum) that are representative for water masses.

	RFI maximum			Average abundance in water mass (%)									
TRF	RFI	Station	Depth	BPLR	ARCT		NADR		NAST				Diss/SD
(nt)	(%)		(m)		1	2	1	2	1	2	out	in	
BPLR													
203	4.3	2	20	3.4	0.9	1.3	0.6	0.5	1.5	1.7	1.1	3.4	2.2
259	6.4	2	20	2.6	0.1	0.9	0.1	0.1	0.3	0.2	0.2	2.6	1.0
Sum				6.0	1.0	2.2	0.7	0.6	1.8	1.9	1.3	5.9	1.4
BPLR_ARCT1													
202	5.8	5	20	4.4	2.6	0.7	0.1	0.1	0.0	0.0	0.2	3.2	1.7
BPLR_ARCT													
249	8.6	6	75	6.9	5.2	5.1	1.3	0.7	1.2	1.3	1.2	5.5	2.4
461/2	4.7	2	20	2.5	1.8	1.5	0.0	0.0	0.0	0.0	0.1	1.7	1.7
sum				9.4	7.0	6.6	1.3	0.7	1.2	1.3	1.2	7.2	2.5
ARCT1													
125	12.3	5	20	1.4	5.5	2.5	3.3	0.6	0.4	0.6	1.5	5.5	1.5
605	4.7	6	20	0.9	2.6	0.2	0.3	0.3	0.3	0.4	0.3	2.6	1.5
sum				2.3	8.1	2.7	3.5	0.8	0.7	1.0	1.8	8.1	1.6
ARCT2													
201	4.8	8	20	0.0	0.0	1.2	0.5	0.0	0.0	0.0	0.1	1.2	0.8
193	17.1	7	40	0.3	3.1	6.3	1.8	2.1	1.3	1.2	2.3	4.8	1.4
158	5.3	7	20	0.0	0.7	1.8	1.3	2.3	0.6	0.0	0.8	1.8	1.2
sum				0.3	3.9	9.3	3.6	4.4	1.9	1.2	2.7	9.9	1.2
ARCT2_NADR													
194	11.9	12	40	0.2	1.1	4.8	4.7	6.6	1.9	0.3	0.9	5.3	2.3
NADR													
195	8.0	12	40	2.7	3.0	0.7	4.1	5.3	1.8	1.3	1.9	4.6	1.7
NADR2													
158	3.5	12	20	0.0	0.7	1.8	1.3	2.3	0.6	0.0	0.8	2.3	1.9
NAST													
183	5.1	16	40	0.1	0.0	0.1	0.1	0.1	1.8	2.0	0.1	1.9	1.3
207	5.1	17	20	0.0	0.0	0.1	0.1	0.1	2.6	2.8	0.2	2.7	1.6
242	4.0	16	75	0.2	0.0	0.1	0.3	0.6	1.8	1.8	0.3	1.8	2.4
sum				0.3	0.0	0.3	0.5	0.8	6.2	6.5	0.5	6.4	1.9
NADR_NAST													
227	5.8	14	75	0.1	0.6	1.1	2.6	2.7	3.2	2.1	0.2	2.6	1.6
246	4.8	13	20	0.0	0.3	0.5	3.0	2.7	3.5	3.9	0.3	3.3	3.1
189	44.1	18	75	1.0	4.1	2.1	19.8	14.7	14.9	22.2	2.8	18.3	1.8
sum				1.2	4.9	3.7	25.4	20.1	21.7	28.2	3.3	24.3	2.2
BPLR_ARCT_NADR													
217	5.3	10	75	2.0	2.0	2.9	2.9	1.3	1.2	0.9	0.9	2.2	1.4
ARCT_NADR_NAST													
204	21.2	12	75	2.1	4.1	4.3	8.5	11.1	7.7	6.0	2.1	6.6	1.1
Deeper water 80–500 m													
152	30.1	13	400	8.0	4.0	5.5	3.8	5.1	1.1	0.8	3.8	19.3	2.4
				11.2	15.6	21.8	17.9	22.2	22.2	18.6			
241	12.9	2	275	2.7	1.5	2.3	0.2	0.2	0.0	0.0	0.9	4.5	1.5
				10.8	5.3	6.5	4.2	3.5	3.3	1.7			

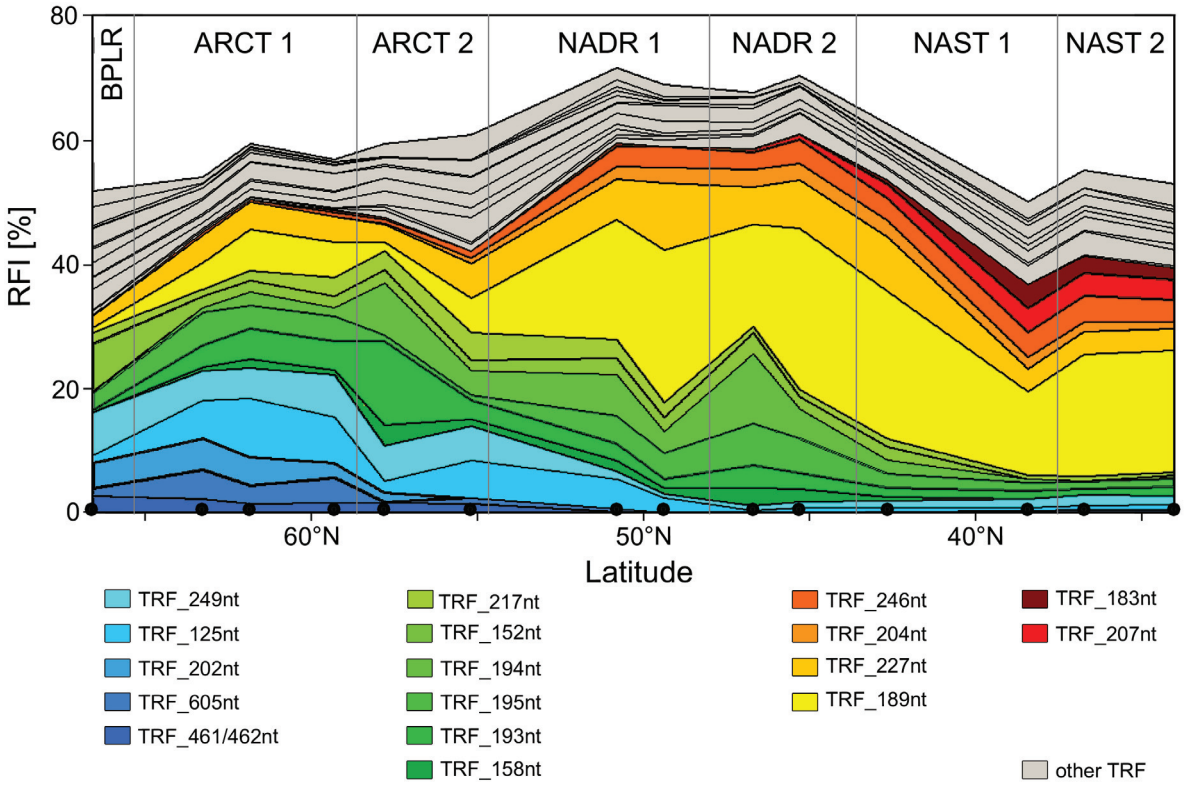

Figure A.3 Regionality of terminal restriction fragments (TRF), represented by their mean (mean of RFI of triplicates) relative fluorescence intensity (RFI) between sea surface and 80 meter depth. Shown are TRFs with a RFI of $\geqslant 4\%$ in at least one water sample. Sampling sites along the transect through the northern (BPLR, ARCT) and southern (NADR, NAST) North Atlanic Ocean provinces are represented by black dots.

dicated a concurrence with the abundance of TRF_125nt, with a Pearson correlation coefficient ρ of 0.89. Within the diversity of 135761 bacterial 16S rRNA sequences present in the dataset, the absence of other iTRF in the range 123–133 nt originating from marine bacteria also supported an assignment of *Synechococcus* iTRF_128nt to the observed TRF_125nt. Ecotypes of *Prochlorococcus* differ in their 16S rRNA genes (Rocap et al., 2002). We found two iTRFs: The iTRF_190nt originated from 16S rRNA gene sequences of *Prochlorococcus* strains that were adapted to high light. Low light adapted *Prochlorococcus* were represented by iTRF_205nt. In the T-RFLP profiles, TRF_189nt and the TRF_204nt showed in the subtropi-

cal province a distribution as expected for high light ($\rho = 0.91$) and low light ($\rho = 0.46$) adapted *Prochlorococcus* (Fig. A.4 2 and 3). *Flavobacteria* had been investigated with 16S rRNA libraries and *in situ* hybridization (Gómez-Pereira et al., 2010). The iTRF_461nt and iTRF_462nt originated from *Polaribacter* only, and iTRF_464nt from *Polaribacter* and the groups NS4 and NS2b. The T-RFLP pattern of TRF_461nt and TRF_462nt concurred in the northern provinces (suppl. Fig. A.S6). *Flavobacteriaceae* VIS4 group was solely responsible for iTRF_604nt. The T-RFLP pattern of TRF_605nt coincided well with the VIS4 population in FISH cell counts of 2% of all DAPI stained cells at the station 4 and 6 (suppl. Fig. A.S6). *Flavobacteriaceae* group DE2 contributed exclusively to iTRF_606nt. DE2 was abundant in ARCT and NAST according to the T-RFLP pattern of TRF_607nt and to FISH cell counts. *Flavobacteriaceae* group DE2 gave also iTRFs in the range of 817 to 825 nucleotides. The TRF_820nt was found only in surface waters in the NAST province, but its abundance was below 1% relative fluorescence intensity. The *in silico* analyses did not allow a clear assignment of other TRFs that were representative for oceanic provinces (Tab. 6.1). In several cases, assignments to *Alphaproteobacteria* as well as *Gammaproteobacteria* were feasible (suppl. Tab. A.S7), an indication for the low taxonomic resolution of the T-RFLP method.

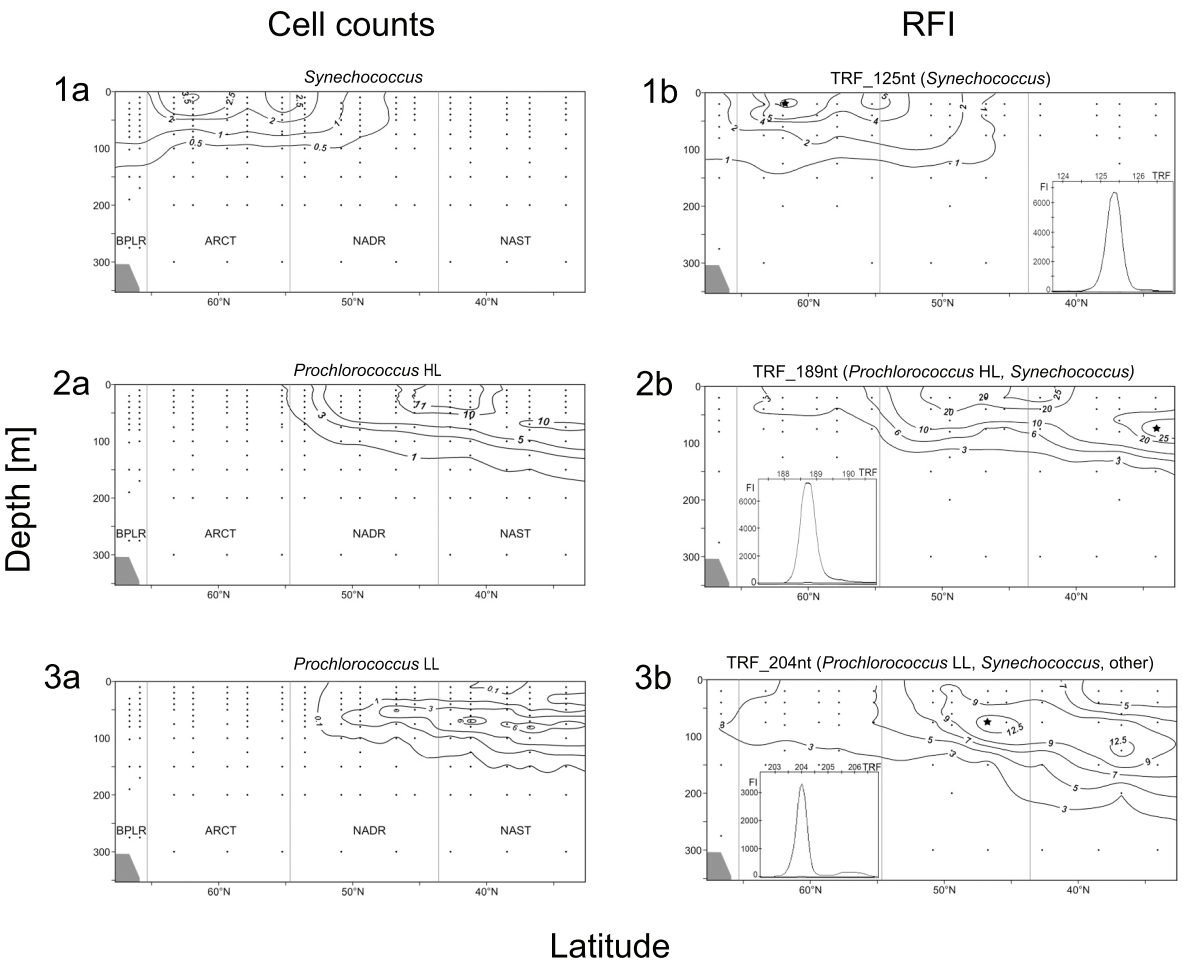

Figure A.4 Cyanobacterial populations visualized by flow cytometry (1a–3a) and by their mean (mean of RFI of triplicates) relative fluorescence intensity (RFI) of affiliating TRFs (1b–3b). Flow Cytometry detected *Synechococcus* (1a) more abundant in the mesotrophic region of the northern North Atlantic Drift and *Prochlorococcus* (2a, 3a) in the oligotrophic central Atlantic gyre. The peak abundance of high light (HL) adapted *Prochlorococcus* ecotype was at 40 meters (2a), whereas the low light adapted (LL) *Prochlorococcus* ecotype was observed at deeper water layers (3a). The pattern of terminal restriction fragments (TRF) corresponded to the related cyanobacterial populations: TRF_125nt affiliated to *Synechococcus* (1b), TRF_189nt affiliated to the HL adapted *Prochlorococcus* ecotype and *Synechococcus* (2b), and, TRF_204nt affiliated to the LL adapted *Prochlorococcus* ecotype, *Synechococcus* and other taxa (3b). A star (*) indicates the sampling site with the largest RFI and the insert shows the corresponding signal in the T-RFLP profile. Contour lines indicate the relative abundance of cell counts (% total cell counts) or TRFs (%RFI). Black dots indicate sampling sites.

Discussion

The biogeography of microorganisms started with the characterization of microbes from Arctic and Antartic (Staley and Gosink, 1999). Diversity studies with reference to the latitude have shown a pole-to-pole biogeography (Ghiglione et al., 2012), a bipolar distribution (Sul et al., 2013) and a latitudinal diversity gradient (Fuhrman et al., 2008). These studies assembled observations from many oceanic provinces, but did not include a concrete transect along one longitude. Baldwin et al. reported the microbial diversity in a Pacific Ocean pole-to-pole transect between 154°W and 172°E and detected four biological provinces: sub-Arctic/Arctic, temperate, tropical, and sub-Antarctic/Antarctic (Baldwin et al., 2005). Each province covered a large range of latitudes. The situation is different in the North Atlantic Ocean, which has four oceanic provinces present on a relative small range of latitudes. This results from the North Atlantic Drift, a profound influence on the history of water masses. Fluorescence in situ hybridization (FISH) (Gómez-Pereira et al., 2010) revealed a biogeography of *Polaribacter* in the northern provinces. Our T-RFLP analyses now showed a biogeography of bacterial populations in the North Atlantic Ocean consistent with water masses along a latitudinal gradient 30°W between 66°39.27'N and 34°24.87'N. The photic pelagial showed large differences between communities, whereas the mesopelagial had less diverse bacterial communities. Larger changes in environmental parameters above the pycnocline as well as the presence of phototrophic microorganisms may contribute to the larger diversity. Unicellular cyanobacteria affiliating to *Synechococcus* and *Prochlorococcus* are among the major bacterial populations with a biogeography in the oceans (Li, 1994; Liu et al., 1997; Veldhuis et al., 1997). The large genetic diversity within *Synechococcus* and *Prochlorococcus* has lead to the definition of ecotypes for genetically well

defined subgroups (Zwirglmaier et al., 2008). *Prochlorococcus* has high light adapted (HL) and low light adapted (LL) ecotypes (Zubkov et al., 2007; Zwirglmaier et al., 2008; Huang et al., 2012). We could assign TRFs to *Synechococcus*, low and high light adapted *Prochlorococcus* ecotypes on the basis of *in silico* fragment length calculations, the biogeographic detection of TRFs and coincidence with fluorometric measurements of pigments. This technique showed the distribution of *Prochlorococcus* ecotypes in different water depth.

In summary, the oceanic provinces in the North Atlantic Ocean hosted different bacterial communities. Bacterial populations varied along the latitudinal transect, so that individual terminal restriction fragments can serve as representative proxy for individual oceanic provinces.

Acknowledgments

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Supporting Information

Variations of pelagic bacterial communities in the North Atlantic Ocean coincide with water masses

Richard L. Hahnke, Christina Probian, Bernhard M. Fuchs and Jens Harder

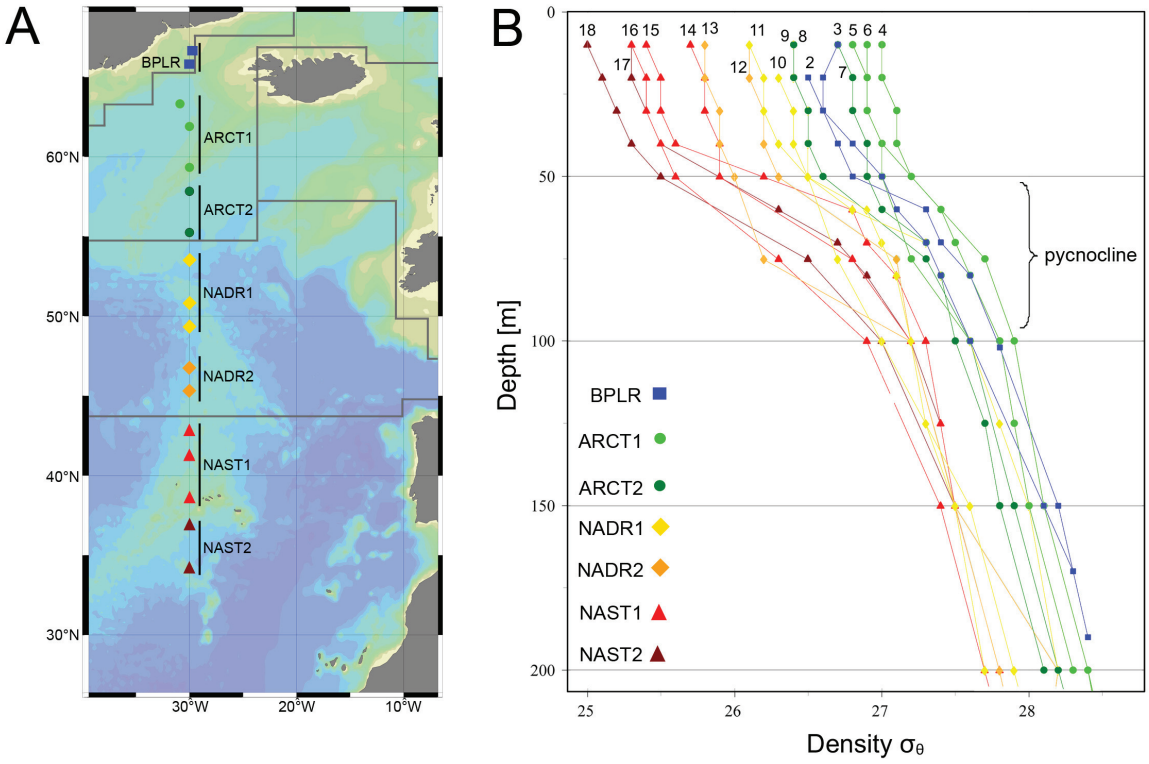

Figure A.S1 Sampling stations and water density of the Merian cruise 03/1 V:I:S:I:O:N, in 2006. (A) Water samples were obtained from the North Atlantic Ocean along the 30°W meridian from the productive cold Greenland current (Boreal Polar, BPLR, 66°39'N) across the cold north (Atlantic Arctic, ARCT) and warm south (North Atlantic Drift, NADR) of the North Atlantic Current to the oligotrophic central Atlantic Ocean (North Atlantic Subtropical Gyre, NAST, 34°24'N). (B) The density (as derived quantity σ_Θ) of the seawater from depths between 20 m and 200 m indicated a pycnocline between 50 and 100 meter.

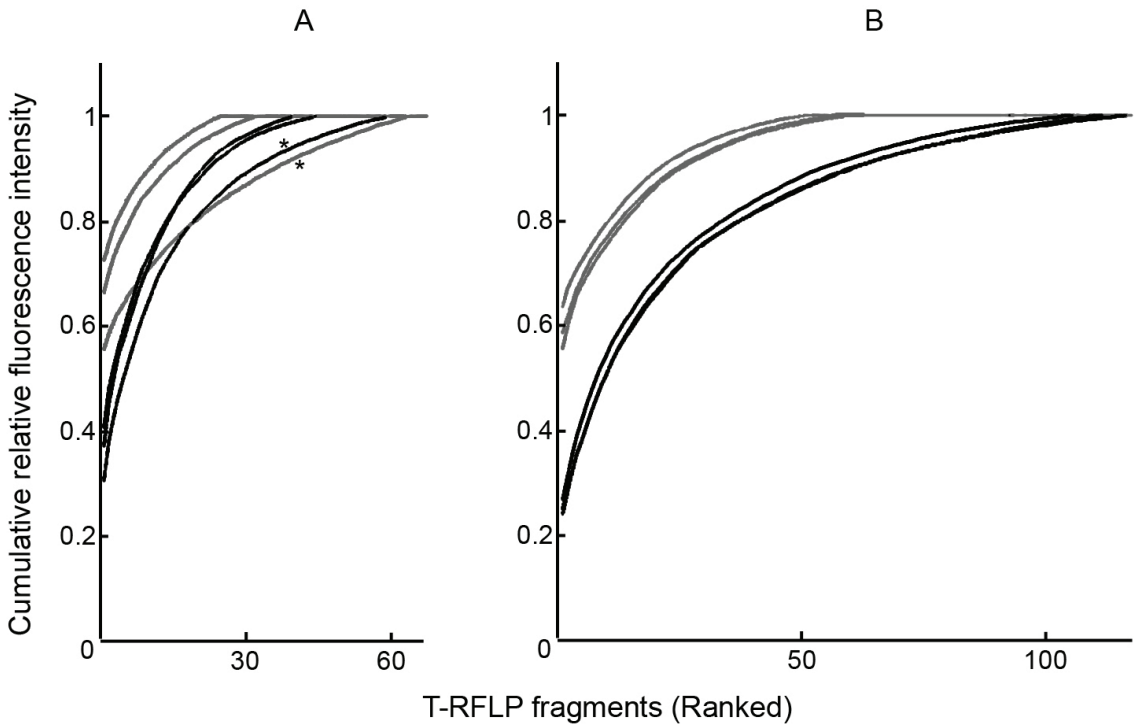

Figure A.S2 Identification of T-RFLP pattern with low phylogenetic information was possible with the k-dominance plot of T-RFLP pattern. The Normalized relative fluorescence intensities were visualized in rank versus cumulated abundance curves. Each line represents the cumulative relative fluorescence intensity of forward (black) or reverse (gray) terminal restriction fragments of one sampling site and their triplicates. The species rank at 100% cumulative abundance (RFI) represents the richness of TRFs of the sampling site. The T-RFLP pattern with a high amount of false positive signals (indicated by a star) originated from a fixed fluorescence intensity threshold and overall low fluorescence intensity in this T-RFLP pattern (A). These T-RFLP patterns were excluded from further analysis. In contrast, the cumulated abundance curves of T-RFLP patterns of comparable good quality had slightly different fluorescence intensity between T-RFLP patterns (B).

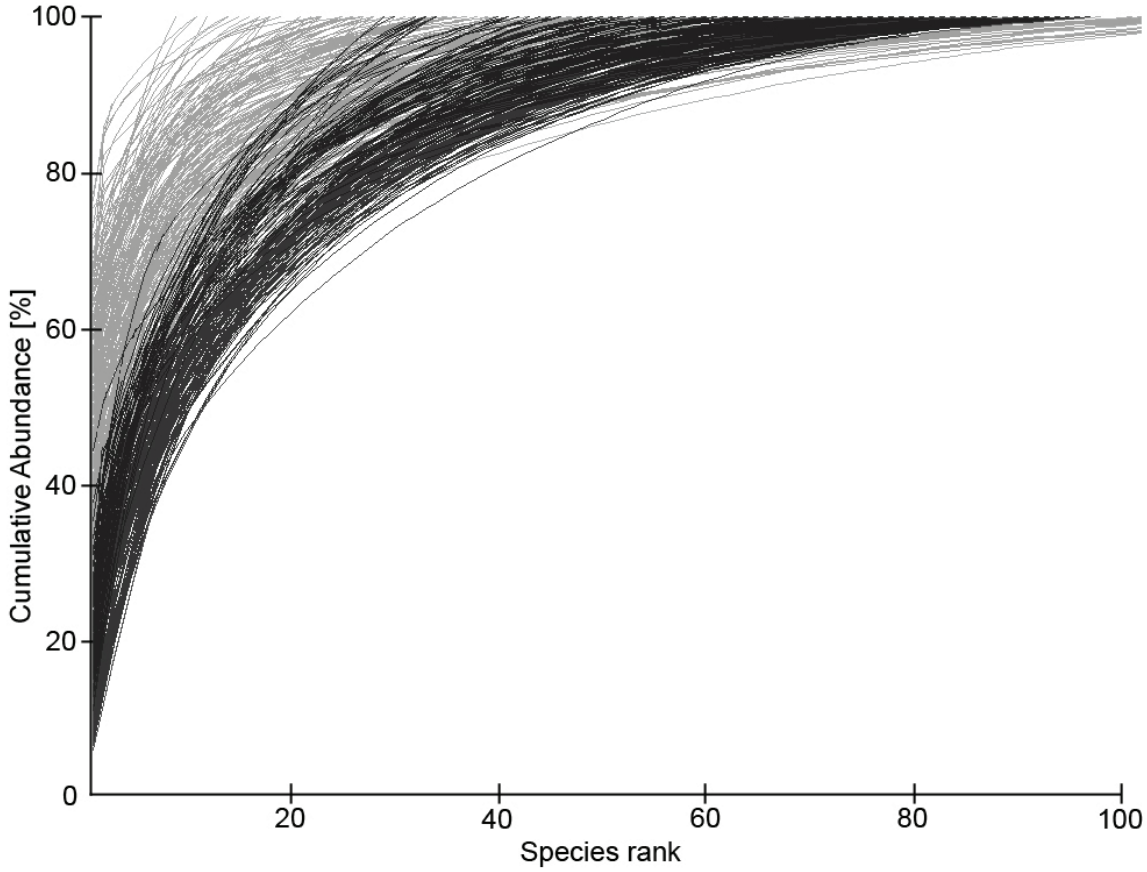

Figure A.S3 The diversity richness is different between terminal restriction fragments derived from the forward (black) and from the reverse (gray) primer. Each black and gray line represents the cumulative relative fluorescence intensity of the terminal restriction fragments of one sample site and their triplicates. The species rank at 100% cumulative abundance (RFI) represents the richness of TRFs of the sample site. TRFs of the reverse primer had a lower species rank compared to the TRFs of the forward primer at the same cumulative abundance. Thus, the richness of the reverse primer TRFs is less than the forward primer TRFs.

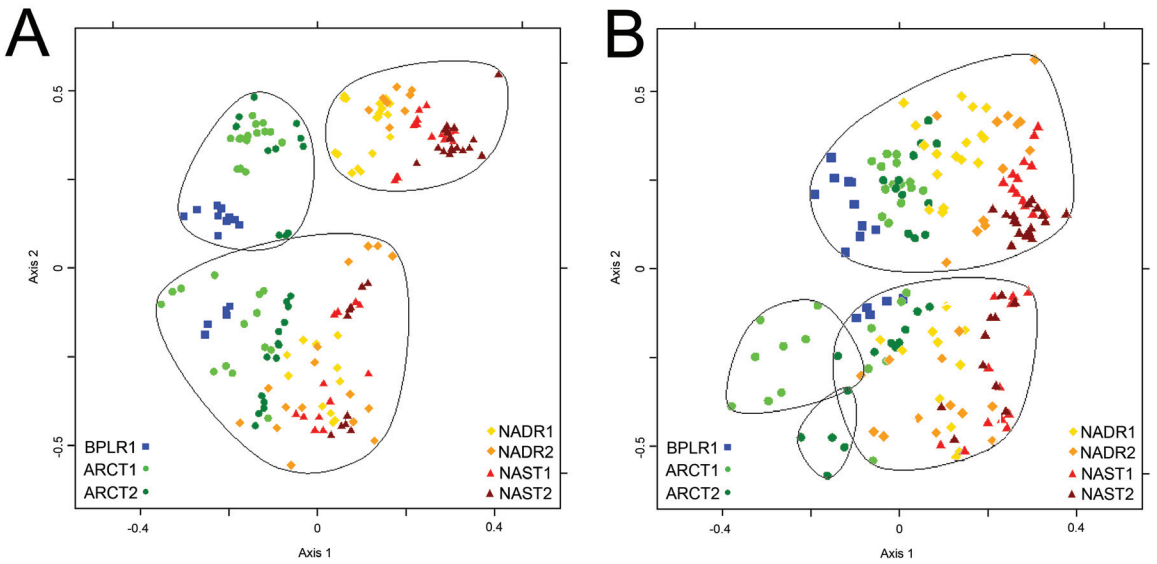

Figure A.S4 The nonmetric multidimensional scaling (nMDS) was applied to visualize (A) the Bray-Curtis similarity based on relative fluorescence intensities, and (B) the Sørensen index (β diversity) based on presents/absents of TRFs between TRF patterns of each sample site. Both biplots had a low stress value of 0.12, indicating a meaningful two dimensional visualization. The presentation of the differences revealed a clustering of sampling sites from one water mass along a latitudinal gradient of the water masses BPLR (square), ARCT (dot), NADR (diamond) and NAST (triangle). A hierarchical clustering defined groups of sampling sites at 48% Bray-Curtis similarity and 55% Sørensen index (indicated by solid gray lines).

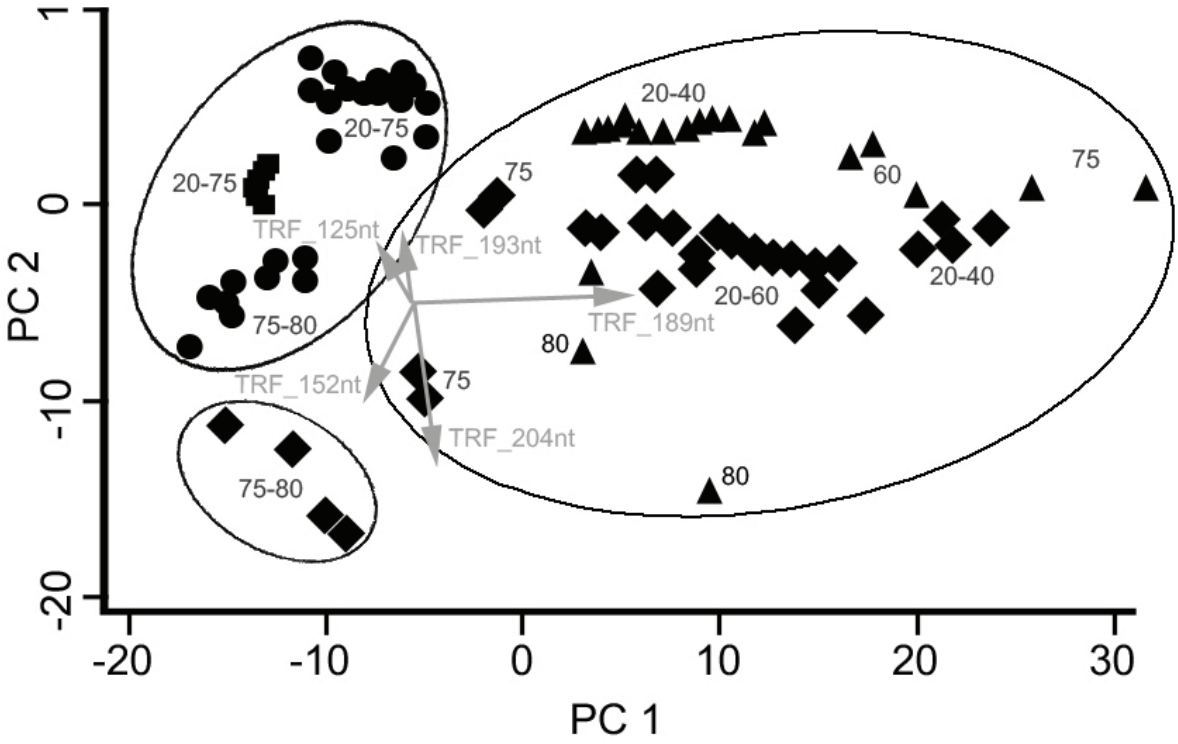

Figure A.S5 The principal component analysis was applied to find terminal restriction fragments (TRF) that cause the changes in the overall community structure. The presentation of the eigenvectors and eigenvalues revealed a differentiation between the northern sample sites, BPLR (black square) and ARCT (black dot), above and below 75 m depth, and in the southern sample sites between NADR (black diamond) and NAST (black triangle), along the first principal component (PC 1). The second principal (PC 2) distinguished in the northern sample sites (BPLR, ARCT) above and below 75 m depth, and in the southern sample sites between NADR and NAST. The TRF_189nt had the largest eigenvector (gray arrow) parallel to the first principal component, meaning a large contribution to the population in the south of the transect. Numbers in gray represent the depth of the sample site. A circle represents sample sites that fall into one hierarchical cluster of 50% similarity.

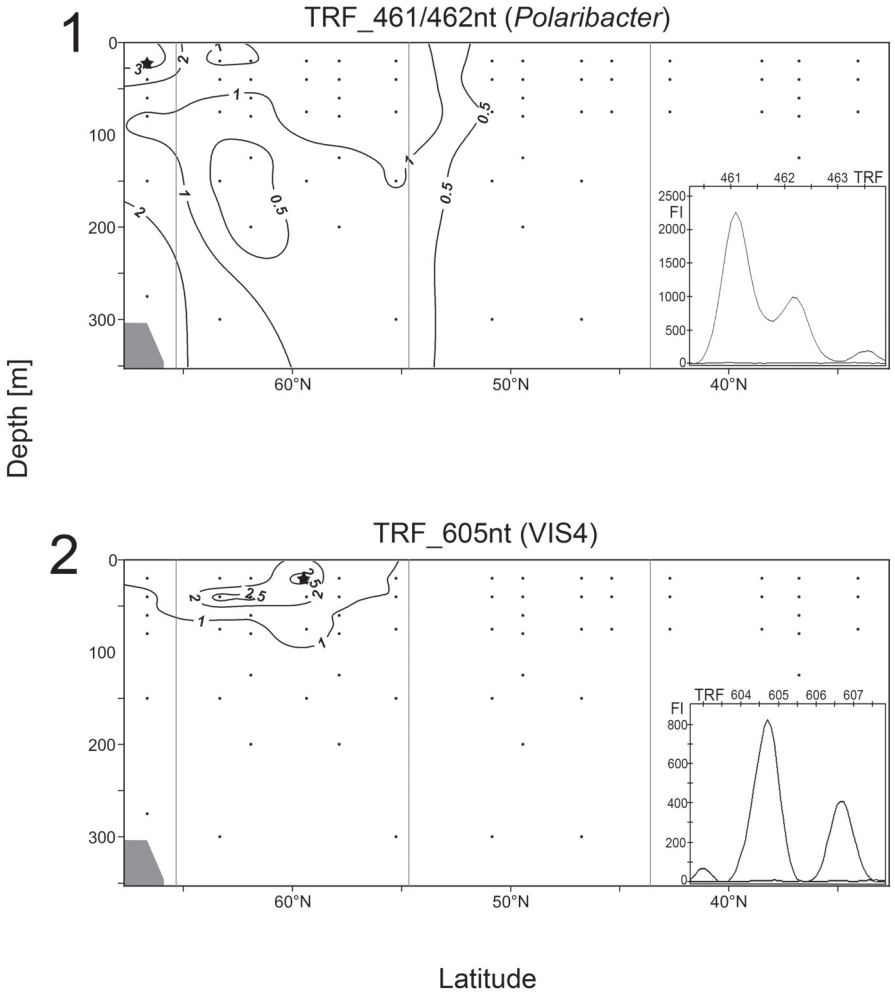

Figure A.S6 Relative fluorescence intensity pattern of significant terminal restriction fragments (TRF) affiliated to the *Flavobacteriaceae Polaribacter* (1) and group NS4 (2). A star (*) indicates the sampling site with the largest RFI and the insert shows the corresponding signal in the T-RFLP profile.

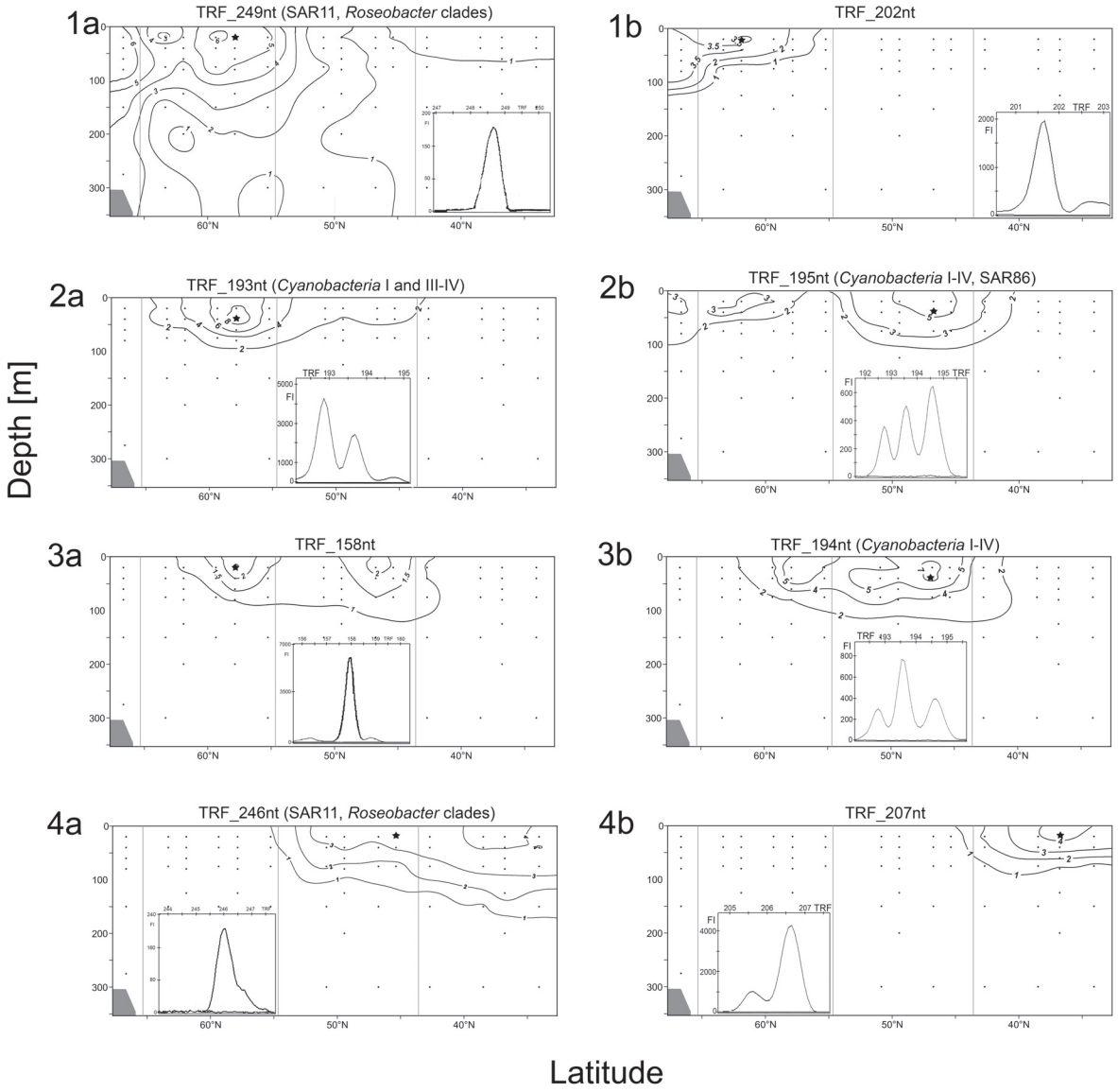

Figure A.S7 Relative fluorescence intensity pattern of terminal restriction fragments (TRF) with a regional distribution and no affiliation to a single taxon in the iTRF calculation. A star (*) indicates the sampling site with the largest RFI and the insert shows the corresponding signal in the T-RFLP profile.

ANOSIM

Analysis of Similarities Two-Way Crossed Analysis

Analysis of similarity (ANOSIM) in PRIMER-E was used to verify the significance of water mass specific clustering of bacterial communities by testing the null hypothesis that bacterial communities from the same water mass were more similar to each other than to bacterial communities in different water masses. To test for differences in bacterial communities between water masses in the epipelagic zone and differences in epipelagic and mesopelagic bacterial communities two-way crossed ANOSIM statistics were generated. ANOSIM statistics were based on the sampling site-similarity matrix of Bray-Curtis coefficients and computed with 999 permutations.

Factor Values

Factor *Water mass*:
BPLR1, ARCT1, ARCT2, NADR1, NADR2, NAST1, NAST2
Factor *Depth*:
upper (above 80 m), deeper (deeper than 80 m)

TESTS FOR DIFFERENCES BETWEEN Water mass GROUPS
(across all upper groups)

Global Test
Sample statistic (Global R): 0.597
Significance level of sample statistic: 0.1%
Number of permutations: 999 (Random sample from a large number)
Number of permuted statistics greater than or equal to Global R: 0

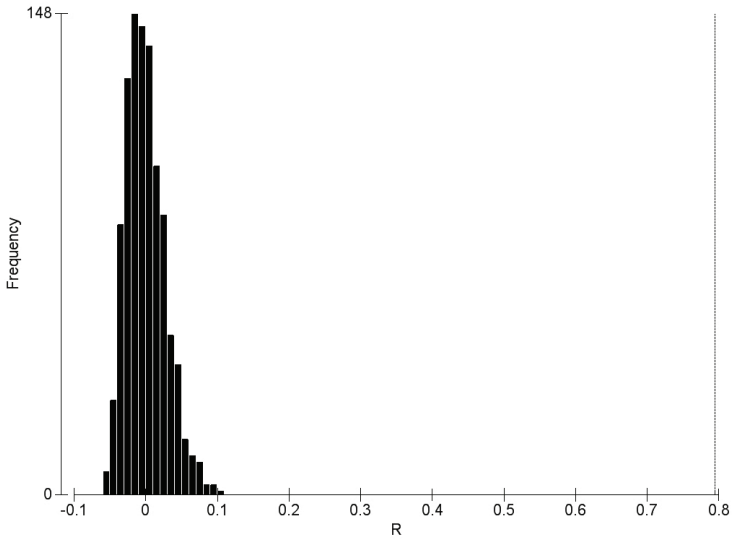

Figure A.S8 Global test for differences between water mass (across all groups) by 2-way-crossed ANOSIM

Pairwise tests

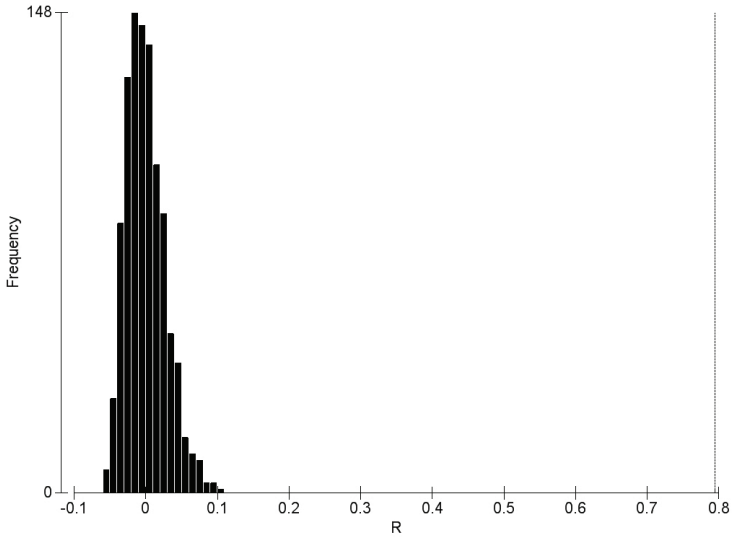

Figure A.S9 Test for differences between water mass, across all water masses above 80 m, by 2-way-crossed ANOSIM

Table A.S1 Result of ANOSIM pairwise tests

Groups	R Statistic	Significance Level	Possible Permutations	Actual Permutations	Number >= Observed
BPLR1, ARCT1	**0.29**	**0.001**	**very large**	**999**	**0**
BPLR1, ARCT2	0.66	0.001	Very large	999	0
BPLR1, NADR1	**0.72**	**0.001**	**very large**	**999**	**0**
BPLR1, NADR2	0.84	0.001	very large	999	0
BPLR1, NAST1	0.99	0.001	very large	999	0
BPLR1, NAST2	0.99	0.001	very large	999	0
ARCT1, ARCT2	**0.321**	**0.001**	**Very large**	**999**	**0**
ARCT1, NADR1	0.49	0.001	very large	999	0
ARCT1, NADR2	0.55	0.001	very large	999	0
ARCT1, NAST1	0.70	0.001	very large	999	0
ARCT1, NAST2	0.78	0.001	very large	999	0
ARCT2, NADR1	**0.577**	**0.001**	**Very large**	**999**	**0**
ARCT2, NADR2	0.61	0.001	very large	999	0
ARCT2, NAST1	0.85	0.001	very large	999	0
ARCT2, NAST2	0.96	0.001	very large	999	0
NADR1, NADR2	**0.225**	**0.002**	**Very large**	**999**	**1**
NADR1, NAST1	0.54	0.001	very large	999	0
NADR1, NAST2	0.69	0.001	very large	999	0
NADR2, NAST1	**0.418**	**0.001**	**Very large**	**999**	**0**
NADR2, NAST2	0.58	0.001	very large	999	0
NAST1, NAST2	**0.20**	**0.001**	**very large**	**999**	**0**

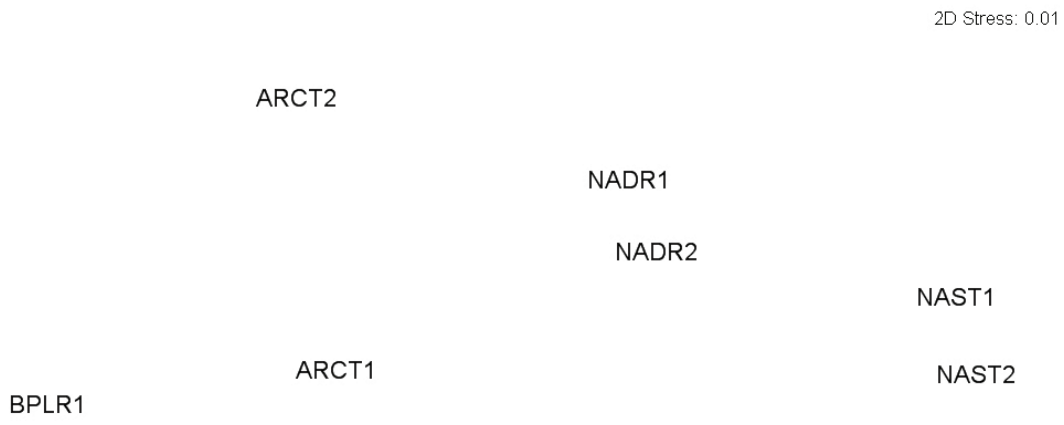

Figure A.S10 2-way-crossed ANOSIM MEANS PLOT - nMDS of R values resemblance

ANOSIM

Analysis of Similarities One-Way Analysis

Analysis of similarity (ANOSIM) in PRIMER-E was used to verify the significance of water mass specific clustering of bacterial communities by testing the null hypothesis that bacterial communities from the same water mass were more similar to each other than to bacterial communities in different water masses. ANOSIM statistics were based on the sampling site-similarity matrix of Bray-Curtis coefficients and computed with 999 permutations.

Factor Values

Factor *Water mass*:
BPLR1, ARCT1, ARCT2, NADR1, NADR2, NAST1, NAST2

TESTS FOR DIFFERENCES BETWEEN water masses
(across all water mass)

Global Test
Sample statistic (Global R): 0.656
Significance level of sample statistic: 0.1%
Number of permutations: 999 (Random sample from a large number)
Number of permuted statistics greater than or equal to Global R: 0

Pairwise tests

Table A.S2 Result of ANOSIM pairwise tests

Groups	R Statistic	Significance Level	Possible Permutations	Actual Permutations	Number >= Observed
BPLR1, ARCT1	**0.29**	**0.001**	**very large**	**999**	**0**
BPLR1, ARCT2	0.66	0.001	very large	999	0
BPLR1, NADR1	0.72	0.001	very large	999	0
BPLR1, NADR2	0.84	0.001	very large	999	0
BPLR1, NAST1	0.99	0.001	very large	999	0
BPLR1, NAST2	0.99	0.001	very large	999	0
ARCT1, ARCT2	**0.32**	**0.001**	**very large**	**999**	**0**
ARCT1, NADR1	0.49	0.001	very large	999	0
ARCT1, NADR2	0.55	0.001	very large	999	0
ARCT1, NAST1	0.69	0.001	very large	999	0
ARCT1, NAST2	0.78	0.001	very large	999	0
ARCT2, NADR1	**0.58**	**0.001**	**very large**	**999**	**0**
ARCT2, NADR2	0.61	0.001	very large	999	0
ARCT2, NAST1	0.85	0.001	very large	999	0
ARCT2, NAST2	0.96	0.001	very large	999	0
NADR1, NADR2	**0.23**	**0.002**	**very large**	**999**	**1**
NADR1, NAST1	0.54	0.001	very large	999	0
NADR1, NAST2	0.69	0.001	very large	999	0
NADR2, NAST1	**0.42**	**0.001**	**very large**	**999**	**0**
NADR2, NAST2	0.58	0.001	very large	999	0
NAST1, NAST2	**0.20**	**0.001**	**very large**	**999**	**0**

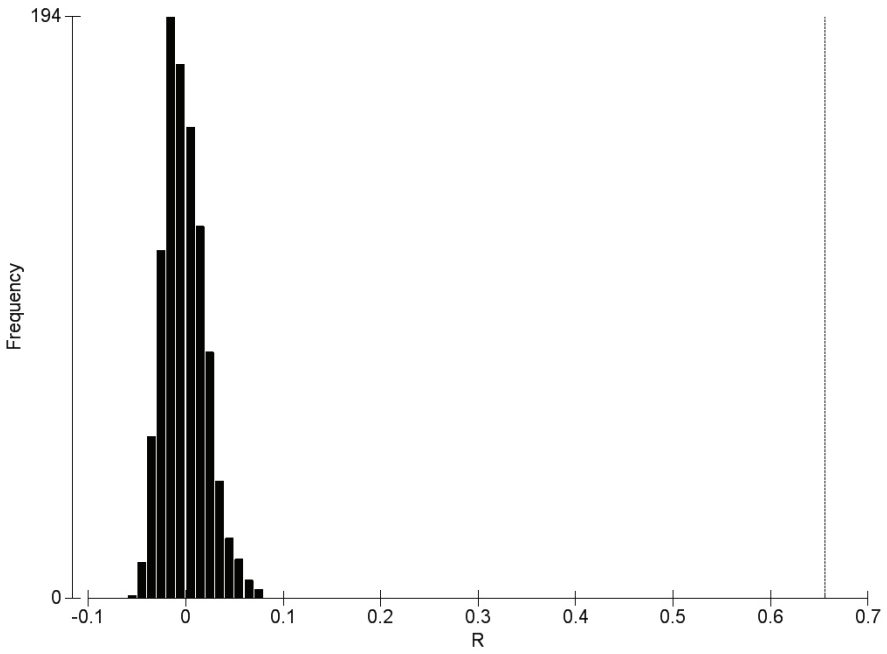

Figure A.S11 Test for differences between water mass, across all water masses above 80 m, by 1-way ANOSIM

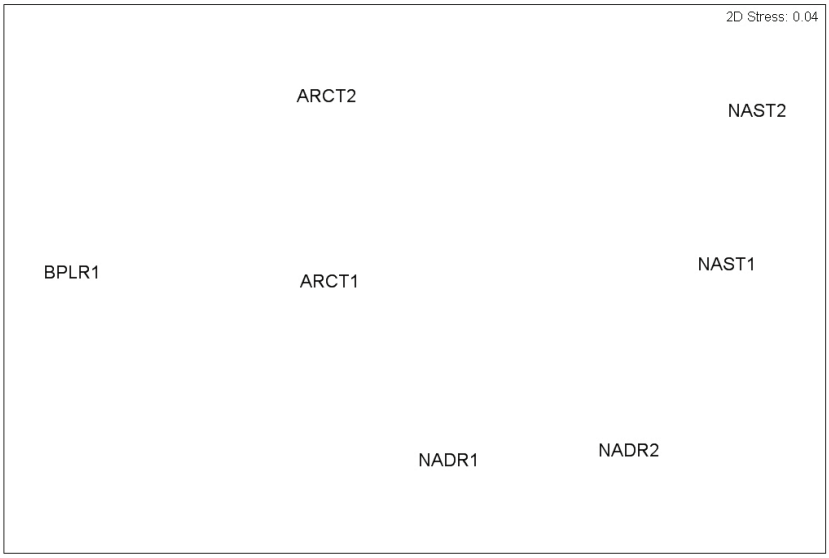

Figure A.S12 1-way ANOSIM MEANS PLOT - nMDS of R values resemblance

References

Aiken, J., Rees, N., Hooker, S., Holligan, P., Bale, A., Robins, D., Moore, G., Harris, R. and Pilgrim, D. (2000). The Atlantic Meridional Transect: overview and synthesis of data. *Prog Oceanogr* **45**, 257–312.

Arnosti, C., Fuchs, B. M., Amann, R. and Passow, U. (2012). Contrasting extracellular enzyme activities of particle-associated bacteria from distinct provinces of the North Atlantic Ocean. *Front Microbiol* **3**, 425.

Baldwin, A. J., Moss, J. A., Pakulski, J. D., Catala, P., Joux, F. and Jeffrey, W. H. (2005). Microbial diversity in a Pacific Ocean transect from the Arctic to Antarctic circles. *Aquat Microb Ecol* **41**, 91–102.

Boström, K. H., Simu, K., Hagström, A. and Riemann, L. (2004). Optimization of dna extraction for quantitative marine bacterioplankton community analysis. *Limnol Oceanogr: Meth* **2**, 365–373.

Bruland, O., Almqvist, E. W., Goldberg, Y. P., Boman, H., Hayden, M. R. and Knappskog, P. M. (1999). Accurate determination of the number of CAG repeats in the Huntington disease gene using a sequence-specific internal DNA standard. *Clin Genet* **55**, 198–202.

Clarke, K. R. (1993). Non-parametric multivariate analysis of changes in community structure. *Aust J Ecol* **18**, 117–143.

Devred, E., Sathyendranath, S. and Platt, T. (2007). Delineation of ecological provinces using ocean colour radiometry. *Mar Ecol Prog Ser* **346**, 1–13.

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J Veg Sci* **14**, 927–930.

Emery, W. J. and Meincke, J. (1986). Global water masses: summary and review. *Oceanol Acta* **9**, 383–391.

Esaias, W. E., Iverson, R. L. and Turpie, K. (2000). Ocean province classification using ocean colour data: observing biological signatures of variations in physical dynamics. *Global Change Biol* **6**, 39–55.

Fuhrman, J. A., Steele, J. A., Hewson, I., Schwalbach, M. S., Brown, M. V., Green, J. L. and Brown, J. H. (2008). A latitudinal diversity gradient in planktonic marine bacteria. *Proc Natl Acad Sci USA* **105**, 7774–7778.

Ghiglione, J. F., Galand, P. E., Pommier, T., Pedrós-Alió, C., Maas, E. W., Bakker, K., Bertilson, S., Kirchman, D. L., Lovejoy, C., Yager, P. L. et al. (2012). Pole-to-pole biogeography of surface and deep marine bacterial communities. *Proc Natl Acad Sci USA* **109**, 17633–17638.

Gómez-Pereira, P. R., Fuchs, B. M., Alonso, C., Oliver, M. J., van Beusekom, J. E. E. and Amann, R. (2010). Distinct flavobacterial communities in contrasting water masses of the North Atlantic Ocean. *ISME J* **4**, 472–487.

Hahn, M., Wilhelm, J. and Pingoud, A. (2001). Influence of fluorophor dye labels on the migration behavior of polymerase chain reaction - amplified short tandem repeats during denaturing capillary electrophoresis. *Electrophoresis* **22**, 2691–2700.

Huang, S. J., Wilhelm, S. W., Harvey, H. R., Taylor, K., Jiao,

N. Z. and Chen, F. (2012). Novel lineages of *Prochlorococcus* and *Synechococcus* in the global oceans. *ISME J* **6**, 285–297.

Li, W. K. W. (1994). Primary production of prochlorophytes, cyanobacteria, and eukaryotic ultraphytoplankton: measurements from flow cytometric sorting. *Limnol Oceanogr* **39**, 169–175.

Liu, H. B., Nolla, H. A. and Campbell, L. (1997). *Prochlorococcus* growth rate and contribution to primary production in the equatorial and subtropical North Pacific Ocean. *Aquat Microb Ecol* **12**, 39–47.

Longhurst, A., Sathyendranath, S., Platt, T. and Caverhill, C. (1995). An estimate of global primary production in the ocean from satellite radiometer data. *J Plank Res* **17**, 1245–1271.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G. et al. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.

Mann, C. R. (1967). Termination of the Gulf Stream and the beginning of the North Atlantic Current. *Deep-Sea Res* **14**, 337–359.

Muyzer, G., Teske, A., Wirsen, C. and Jannasch, H. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**, 165–172.

Olejniczak, M., Kozlowski, P., Sobczak, K. and Krzyzosiak, W. J. (2005). Accurate and sensitive analysis of triplet repeat expansions by capillary electrophoresis. *Electrophoresis* **26**, 2198–2207.

Oliver, M. J. and Irwin, A. J. (2008). Objective global ocean biogeographic provinces. *Geophys Res Lett* **35**, 1–6.

Platt, T. and Sathyendranath, S. (1999). Spatial structure of pelagic ecosystem processes in the global ocean. *Ecosystems* **2**, 384–394.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W. G., Peplies, J. and Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**, 7188–7196.

Ramette, A. (2009). Quantitative community fingerprinting methods for estimating the abundance of Operational Taxonomic Units in natural microbial communities. *Appl Environ Microbiol* **75**, 2495–2505.

Rocap, G., Distel, D. L., Waterbury, J. B. and Chisholm, S. W. (2002). Resolution of *Prochlorococcus* and *Synechococcus* ecotypes by using 16S-23S ribosomal DNA internal transcribed spacer sequences. *Appl Environ Microbiol* **68**, 1180–1191.

Schattenhofer, M., Wulf, J., Kostadinov, I., Glöckner, F. O., Zubkov, M. V. and Fuchs, B. M. (2011). Phylogenetic characterisation of picoplanktonic populations with high and low nucleic acid content in the North Atlantic Ocean. *Syst Appl Microbiol* **34**, 470–475.

Schlitzer, R. (2002). Interactive analysis and visualization of geoscience data with Ocean Data View. *Comput Geosci* **28**, 1211–1218.

Staley, J. T. and Gosink, J. J. (1999). Poles apart: biodiversity and biogeography of sea ice bacteria. *Annu Rev Microbiol* **53**, 189–215.

Sul, W. J., Oliver, T. A., Ducklow, H. W., Amaral-Zettler, L. A. and Sogin, M. L. (2013). Marine bacteria exhibit a bipolar distribution. *Proc Natl Acad Sci USA* **110**, 2342–347.

Tarran, G. A., Heywood, J. L. and Zubkov, M. V. (2006). Latitudinal changes in the standing stocks of nano- and picoeukaryotic phytoplankton in the Atlantic Ocean. *Deep-Sea Res Pt II* **53**, 1516–1529.

Teeling, H., Fuchs, B. M., Becher, D., Klockow, C., Gardebrecht, A., Bennke, C. M., Kassabgy, M., Huang, S., Mann, A. J., Waldmann, J. et al. (2012). Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* **336**, 608–611.

Ter Braak, C. J. F. (1986). Canonical correspondance analysis: a new eigenvector technique for multivariate direct gradient analysis. *Ecology* **67**, 1167–179.

Veldhuis, M. J. W., Kraay, G. W., van Bleijswijk, J. D. L. and Baars, M. A. (1997). Seasonal and spatial variability in phytoplankton biomass, productivity and growth in the northwestern Indian Ocean: The southwest and northeast monsoon, 1992–1993. *Deep-Sea Res Pt I* **44**, 425–449.

Zubkov, M. V., Mary, I., Woodward, E. M. S., Warwick, P. E., Fuchs, B. M., Scanlan, D. J. and Burkill, P. H. (2007). Microbial control of phosphate in the nutrient-depleted North Atlantic subtropical gyre. *Environ Microbiol* **9**, 2079–2089.

Zwirglmaier, K., Jardillier, L., Ostrowski, M., Mazard, S., Garczarek, L., Vaulot, D., Not, F., Massana, R., Ulloa, O. and Scanlan, D. J. (2008). Global phylogeography of marine *Synechococcus* and *Prochlorococcus* reveals a distinct partitioning of lineages among oceanic biomes. *Environ Microbiol* **10**, 147–161.

Curriculum vitae

First name	Richard
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Oct 2009– Jun 2013	**PhD candidate (Dr. rer. nat.) in Biology** at the University of Bremen, Germany
Oct 2007– Jul 2009	**M.Sc. in Molecular Biology** at the University of applied sciences Gelsenkirchen, Germany "Analysis of structure and function of microbial populations of the South Pacific sediment."
Oct 2004– Aug 2007	**B.Sc. in Molecular Biology** at the University of applied sciences Gelsenkirchen, Germany "Determination of T-RFLP fragment sizes."
Aug 2002– May 2003	**Diploma qualifying for the admission to university of applied sciences** at the College of Further Education, Berlin-Neukölln, Germany
Aug 1999– May 2001	**Education as Biological-technical assistant** at the College of Further Education, Werder (Havel), Germany

Publications

In press

Hahnke, R. and Harder, J. (2013). Phylogenetic diversity of *Flavobacteria* isolated from the North Sea on solid media. *Syst Appl Microbiol* **in press**

Hahnke, R. L., Probian, C., Fuchs, B. M. and Harder, J. (2013). Variations of pelagic bacterial communities in the North Atlantic Ocean coincide with water bodies. *Aquat Microb Ecol* **in press**

Mann, A. J., Hahnke, R. L., Huang, S., Werner, J., Xing, P., Barbeyron, T., Huettel, B., Stüber, K., Reinhardt, R., Harder, J. et al. (2013). The genome of the algae-associated marine flavobacterium *Formosa agariphila* KMM 3901^{T} reveals a broad potential for the degradation of algal polysaccharides. *Appl Environ Microbiol* **in press**

In preparation

Hahnke, R. L., Bennke, C. M., Fuchs, B. M., Mann, A. J., Teeling, H., Amann, R. and Harder, J. (2014). Dilution cultivation of marine heterotrophic bacteria benefiting from a coastal diatom bloom. *Environ Microbiol* **in prep.**

Xing, P., Hahnke, R. L., Mann, A. J., Werner, J., Unfried, F., Schweder, T., Harder, J., Amann, R. and Teeling, H. (2013). Niche separation of two *Polaribacter* strains isolated from Helgoland, North Sea. *Environ Microbiol* **in prep.**

Scientific Meetings and Conferences

Mar 2013 Talk	**VAAM Annual Meeting**, Bremen, Germany. 'Dilution cultivation yielded novel psychrophilic marine bacteria, representatives of the phytoplankton decomposing community'
Jan 2013 Invited talk	**Microbiological Colloquium**, ICBM, Oldenburg, Germany. 'Cultivation of novel marine bacteria and their ecological niches during phytoplankton decay'
Aug 2012 Poster	**14th ISME Conference**, Copenhagen, Denmark. 'Culturability and ecophysiology of marine microorganisms associated with a phytoplankton bloom'
May 2012 Talk	**Scientific Advisory Board**, MPI for Marine Microbiology, Bremen, Germany. 'Growth and physiology of cultivable abundant bacteria from the North Sea phytoplankton spring bloom'
Jul 2011 Poster	**MIMAS-Symposium**, Greifswald, Germany. 'Cultivation of marine bacteria of significant abundance at low cell density'
Apr 2011 Poster	**VAAM Annual Meeting**, Karlsruhe, Germany. '*Flavobacteria* of the North Sea: Diversity of Culturability'
Mar 2010 Poster	**VAAM Annual Meeting**, Hannover, Germany. 'Microbial populations of manganese oxidizing enrichment cultures of the South Pacific Gyre sediment'
Mar 2009 Poster	**VAAM Annual Meeting**, Bochum, Germany 'Identification of local microbial communities in the North Atlantic Ocean by T-RFLP'

Erklärung der selbstständigen Erarbeitung

Erklärung gemäß §6 Abs. 5 der Promotionsordnung der Universität Bremen für die mathematischen, natur- und ingenieurwissenschaftlichen Fachbereiche

Hiermit versichere ich, dass ich die vorliegende Dissertation mit dem Titel "Cultivation of *Flavobacteria* and other *in situ* abundant bacteria from the North Sea"

1. ohne unerlaubte fremde Hilfe angefertigt habe
2. keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe und
3. die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Bremen, 24. Mai 2013 _____

(Richard Hahnke)

Bremen, 2013